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ROY WALDO MINER

BIOFLAVONOIDS AND THE CAPILLARY

BY

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* This series of papers is the result of a conference on *Bioflavonoids and the Capillary* held by the Section of Biology of The New York Academy of Sciences, February 11, 1955.

Part I. Laboratory Studies

CHEMISTRY OF BIOFLAVONOIDS

By Willard E. Baier
Research Department, Sunkist Growers, Ontario, Calif.

It is odd that the term "flavonoid" did not appear until 1949, if one considers that the group name "carotenoids" had long been used among biochemists. Flavonoids comprise the broad group of compounds of carbon-hydrogen-oxygen including the flavones, flavonols, flavanones, and their derivatives. The chalcone forms of the natural flavanones and certain derivatives of them are also included.

The term bioflavonoid is of even more recent origin. Subsequent to 1936, when vitamin P, or citrin, was announced by Szent-Györgyi and its flavonoid nature was determined, some controversy developed as to whether "vitamin P" met all the classical requirements of a vitamin even though nutritionally and therapeutically useful. To avoid this needless controversy, "bioflavonoid" was introduced into the literature to designate those flavonoids having biological activity. The term was first suggested by B. L. Oser and first used in the literature by E. F. Bryant. Thus, by this terminology, "vitamin P," first investigated by Szent-Györgyi, was a crude citrus bioflavonoid.

The purpose of this very brief discussion is to set forth some of the more important chemical and physical characteristics of those flavonoids which have achieved some prominence as pharmaceuticals (or, we might say, as bioflavonoids) and to touch on their commercial production and availability.

The basic unit of the flavones is the γ-pyrone ring occurring as phenylbenzopyrone, in which there are various substitutions (phenolic OH groups) for the hydrogen atoms. Most frequently, in the flavonoids derived from natural sources, these substitutions are to be found in 3, 5, 7, 3′ and 4′ positions. Most frequently, also, they occur as glycosides, the sugars usually being attached at the 7 position.

When an OH group substitutes for the H in the 3 position of the pyrone ring, the flavone is termed a flavonol. When the double bond at this position in the original flavone is saturated with H atoms, the substance becomes a flavanone.

Drastic reduction of the carbonyl of the pyrone ring, as with magnesium in acid alcohol, produces another class of compound, anthocyanidin, and subsequent saturation of this ring with hydrogen gives us catechin.

It will be seen from the foregoing that we are taking some liberty with the term "flavonoid or bioflavonoid" when we include the diverse chemical structures represented in these various groups of compounds. They are compatible, however, with the broader designation considered by Geissman with reference to biogenesis of flavonoid compounds, namely the term "C_6—C_3—C_6— compounds" since bioflavonoids, in their sugar-free or aglycone forms, are composed of two six-carbon fragments joined by a chain of three-carbon atoms.

The flavonoids are widely distributed in nature. Flavones constitute the

majority of the natural pigments which have been used as yellow dyestuffs. Named from the Latin for yellow, many flavones occur not only in yellow flowers but in many white ones, where the flavone's presence may be demonstrated by the yellow color reaction with alkalies. Incidentally, Doctor Szent-Györgyi used and reported a somewhat more specific alkali red color reaction in the case of the first so-called vitamin P bioflavonoid derived from lemons, a bioflavonoid still not unequivocally identified.

Another color reaction is of interest at this point. Before Doctor Szent-Györgyi's discovery of the vitamin P effect, C. W. Wilson had observed in our laboratories, under certain conditions, the development of a yellow color upon reacting lemon juice solids with boric acid. No lemon constituent then known would account for this color reaction, which was later determined to distinguish readily certain flavones and chalcones from nonreacting flavanones. Thus, perhaps, did Wilson have a color reaction for vitamin P a few years before vitamin P was discovered.

The alkali, the boric acid, and the cyanidin tests, all comparatively simple color reactions, serve to demonstrate the almost universal distribution of the flavonoids. Fruits, tree barks, flowers, vegetables, and tobacco are among the important flavonoid sources. Again, as mentioned in the earliest publications, general occurrence of flavonoids, including feed and bedding materials, may account for sufficient quantities in animal experiments to nullify significance of the control group. As a dietary source of the bioflavonoids, the citrus fruits probably are the most important, considering the amounts of these fruits consumed, their bioflavonoid contents, and possibly, in the case of lemon, specificity of the substance involved.

There are marked variations in physical properties between the several chemical groups among the flavonoids, as would be expected. Less expected is the situation prevailing within a single group. For example, the flavanone hesperidin derived from oranges and lemons and the flavanone naringin derived from grapefruit are very similar chemically. Both are rutinosides, that is, the glycoside is rhamnoglucose at the seven position. They differ only in that the hesperidin is 3′ hydroxy, 4′ methoxy, whereas the naringin contains only the hydroxy substituent at the 4′ position. Hesperidin is soluble in such biological fluids *in vivo* as are readily absorbed by the animal from ingested sources. When we come to pure water *in vitro*, the solubility of hesperidin, either hot or cold, is extremely slight, almost nil, whereas the usual form of naringin is soluble to an extent of about 0.1 per cent in cold water and many times more soluble hot. There is a metastable form of naringin which was found by C. W. Wilson to be soluble to an extent of perhaps 10 per cent in cold water, but no corresponding hesperidin modification.

When we come to the aglycones of hesperidin and naringin, we encounter another peculiarity. Naringenin is less soluble than its glycoside, naringin, which is expected, but hesperetin is more soluble than its glycoside, hesperidin, for which no reason appears.

The chalcone forms of hesperidin and naringin, resulting from treatment at high pH, may be more soluble than the flavanone forms when compared in acid

or neutral solution, but ring closure takes place rapidly under these conditions, with reversion to the flavanones. If, however, the chalcones are partially methylated while on the alkaline side, ring closure is prevented even after acidification. Both hesperidin methyl chalcone and naringin methyl chalcone are extremely soluble, almost infinitely soluble, whereas the corresponding aglycones, partially methylated, have very limited solubilities. Still another anomaly is the fact that the ethylated chalcones, whether glycosides or aglycones, are insoluble.

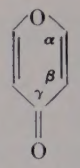
Enough has been said to make clear that no consistent behavior is shown by individuals of a group or, therefore, by the groups which have been enumerated as flavonoids. The latter are like flavones in general chemical structure, but it is certainly too much to expect that they be totally consistent in physical and all chemical properties.

Methods of recovering the bioflavonoids from the source materials used in commercial production usually fall into three classes depending upon the extraction solvent. It may be of interest to note, first, what might be considered a fourth method, the earliest method we employed. This method is actually a mechanical one involving merely the removal of solid hesperidin from metal surfaces on which it had accumulated. When fresh orange or lemon materials are continuously flowing over metal surfaces, a very dense deposit of hesperidin builds up. This deposit holds tenaciously to the metal and survives ordinary cleaning processes. It is common practice now to clean all such surfaces with detergents sufficiently alkaline to dissolve the hesperidin but formerly, in the case of equipment used in the manufacture of by-products, the hesperidin was allowed to deposit until it had reached a substantial thickness, perhaps 5 mm. or so, and it was then manually chipped off with a chisel and hammer. Very soon after the first publications concerning the so-called citrin, of which hesperidin was reported to be a component, there was a demand for hesperidin for experimental purposes. Hesperidin recovered by this mechanical process served to take care of the immediate needs until the extraction processes could be set up.

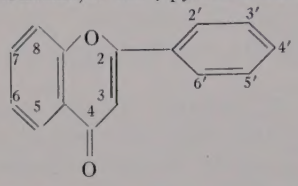
The extraction processes employ as solvents aqueous alkali, hot water, or a water-miscible solvent, usually isopropanol.

The alkaline agents most commonly used are sodium hydroxide or calcium hydroxide, with which the fresh citrus peel is macerated. The solution is pressed out and clarified, and the hesperidin is precipitated by acidification. There are various modifications, depending upon what other disposition is to be made of the peel and the purity of hesperidin desired and, hence, whether or not the material is to be repurified by putting it through a similar cycle a second time. In this connection, we should call attention to a fact well known by those who have worked with the flavonoids—flavonoids have a remarkable tendency to form complexes with each other that considerably alter the apparent solubility. Thus hesperidin, which is almost completely insoluble in its pure form, can be held in aqueous solution in the form of some of its natural complexes with other citrus flavonoids. The hesperidin will then slowly crystallize from the solution, even after the latter has been filtered brilliantly clear.

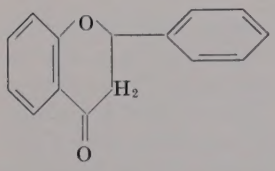
Typical Structures of Some Flavonoid Substances



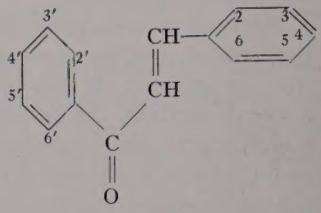
Most of the flavonoid substances are derivatives of γ-pyrone and, in particular, of the γ-pyrone derivative, 2-phenyl benzopyrone.



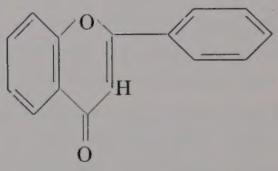
Structure Types



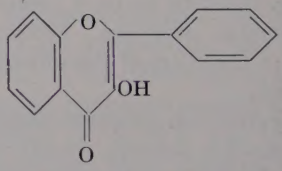
Flavanone



Chalcone Nucleus



Flavone



Flavonol

Glycosides and Aglycones

The sugar-free flavonoids are called aglycones, while the term "glycoside" is applied when sugars are attached to the molecule.

Qualitative Reactions

Cyanidin (Willstäter. 1914. Ber. **47:** 2874.). 0.1 g. flavonoid substance in test tube in 10 ml. 50% C_2H_5OH and 2 ml. conc. HCl. Add 0.13 g. magnesium (10 cm. strip 3 mm. wide, usually). Cool and dilute with an equal volume of water. Colors from orange to purple indicate presence of flavonoids.

Aglycone (Bryant. 1950. J. Am. Pharm. Assoc., Sci. Ed. **39:** 480.). Make test as for cyanidin except at end add 3 ml. octyl alcohol and shake tube vigorously. If color dissolves in octyl alcohol, compound tested is the aglycone.

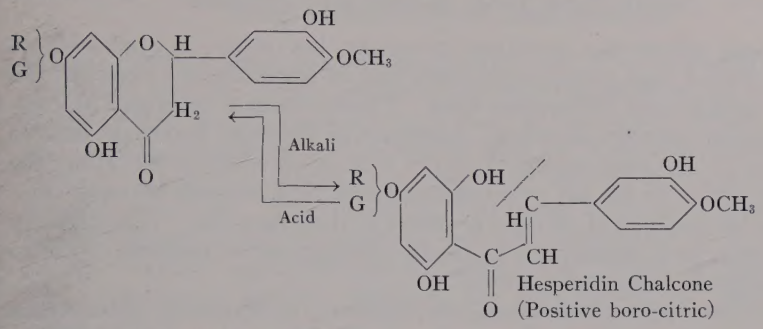
Boro-Citric (Wilson. 1939. J. Am. Chem. Soc. **61:** 2303–6.). Yellow color in presence of boric acid in anhydrous acid solution indicates certain structural types.

Figure 1

Glucoside — Aglycone

FLAVANONES (positive cyanidin and negative boro-citric tests).

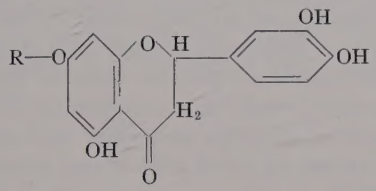
Hesperidin



Hesperetin

Hesperidin Chalcone (Positive boro-citric)

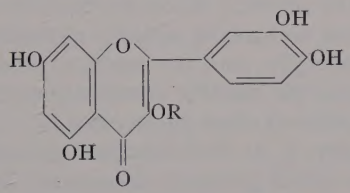
Eriodictyol Glycoside



Eriodictyol

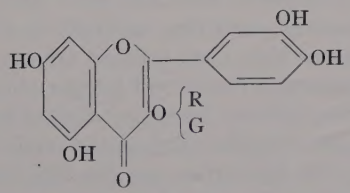
FLAVONOLS (positive cyanidin and positive boro-citric tests).

Quercitrin



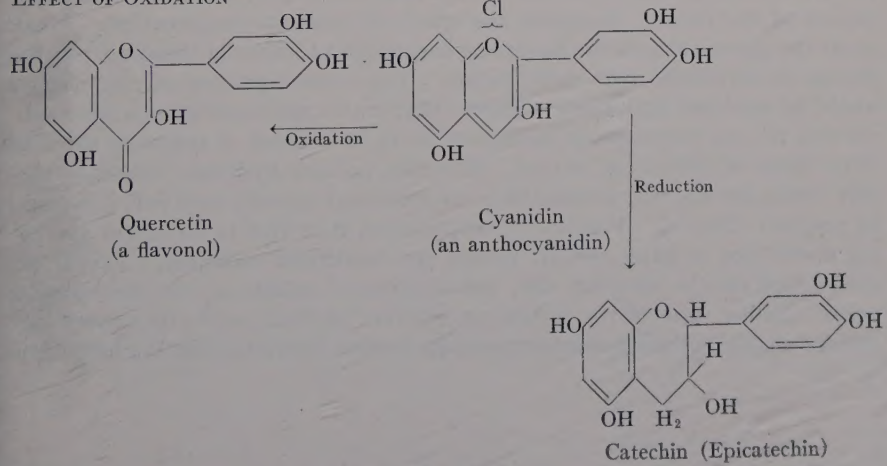
Quercetin

Rutin



Quercetin

EFFECT OF OXIDATION



Quercetin (a flavonol)

Cyanidin (an anthocyanidin)

Catechin (Epicatechin)

FIGURE 2

The second solvent for extraction processes, hot water, is applicable to flavonoids with a high temperature coefficient of solubility, for example, the flavanone naringin and the flavanol rutin. The flavonoid is recovered by cooling the solution after hot filtration. Inasmuch as the more important sources of materials are also good sources of pectin, it is understandable that either the alkaline extraction or the hot water extraction of the flavonoids becomes complicated by the presence of pectates or pectin in these extracts. This difficulty can be avoided to a considerable extent by allowing native enzymes to act to convert the pectin to insoluble forms.

Contamination with pectic substances, however, is frequently encountered unless the solution is repurified or unless the bioflavonoids are extracted by the solvent method. Isopropanol is a fair solvent for several bioflavonoids and is a precipitant for pectin. Methanol, acetone, and ethanol are others.

Potentially, many derivatives are possible from the bioflavonoids. The purpose of chemical derivatives would be to enhance the pharmacological properties or, at least, to improve solubility and other such physical properties. It was with the latter purpose in mind that many investigators have studied modifications of hesperidin.

These attempted modifications include (1) the natural soluble complex as it exists in the fruit; (2) the physical chemical solubilization with solvents; and (3) chemical modifications, such as the partially methylated chalcone of hesperidin, phosphorylated hesperidin, and the reaction product of hesperidin with ethylchlorocarbonate, also the metallic combinations and chelated complexes which are often simply referred to as the flavonates.

Under the first category, J. A. Hall described glucose-hesperidin complexes which were soluble, but which gradually dissociated to precipitate hesperidin from solution. Wawra and Webb described what they believe to be a soluble complex of hesperidin chalcone occurring naturally in the fruit. To date, such natural soluble complexes as have been isolated are not stable.

The solubilizing agents used in the second category have included such agents as glycol and other obvious solvent mixtures, dialkyl amines or salts of amino or aminosulfonic acids, and colloidal saccharated ferric oxide.

In the third category, the derivatives are described in the patent literature. We can discuss briefly hesperidin methyl chalcone which, while not necessarily typical of the others, illustrates the effect of chemical modification. Alkali opens the pyrone ring of the flavanone hesperidin to form the chalcone, but the process is reversible upon acidification. The reverse process of ring closure would be rendered impossible, theoretically, if two methoxyl groups were substituted for the hydroxyls so positioned as to be capable of restoring the ring when either of them is so reacted. Actually, because hydrogen bonding probably limits the reaction possibilities, one methoxyl appears sufficient if it could be properly directed. Practically, methylation is carried to an extent averaging about two to three methyl groups per hesperidin molecule. This is accomplished on the alkaline side, using dimethyl sulfate as the methylating agent. Saline wash of the salted-out reaction product serves to remove any sodium methyl sulfate, and isopropanol extraction then separates the hesperidin

methyl chalcone from the sodium chloride and other impurities. I stated previously that the product is almost infinitely soluble in water, and that it is stable at high temperatures in a wide range of pH values, so that its physical and chemical properties now become suitable for parenteral administration.

Many flavonoids, including those we have touched upon, are available in research quantities. The commercially available citrus bioflavonoids are hesperidin; hesperidin methyl chalcone; naringin, particularly for its bitterness as a flavor modifier; lemon bioflavonoid complex; and calcium flavonate glycoside. The bioflavonoids esculine, quercetin, and rutin are derived from other than citrus sources.

In summary, the bioflavonoids comprise several groups of biologically active flavonelike compounds having different physical and chemical properties. They are widely distributed in nature, citrus fruits being important dietary and pharmaceutical sources.

BIOCHEMISTRY OF THE BIOFLAVONOIDS

By Gustav J. Martin
Research Laboratories, The National Drug Co., Philadelphia, Pa.

It is to be expected that compounds of a flavonoid structure should manifest a relatively high degree of biological activity. The chemical nature of the flavonoid molecule, containing, as it does, reactive hydroxyl and carbonyl substitutes, would suggest such activity. The ability of compounds of this type to chalconize and to form quinones in biological systems, with the formation of structures known to interact with specific enzymic functional groups, is also indicative. It is therefore not surprising that there are a number of reports in the literature linking inhibitory action of the flavonoids against specific enzymes with specific elements of their chemical structure.

For example, Bartlett, in 1948,[2] reported inhibition of succinoxidase by hydroxylated chalcones. He linked this inhibitory effect directly to the potentiality for quinone formation of the chalcones tested, with a reaction between the formed quinones and the sulfhydryl groups of the enzyme. Beiler and his coworkers, in 1950,[3] reported similar findings with choline acetylase. In this system, maximal activity was found to be exerted by compounds having a 3,4-dihydroxyl grouping. Ease of chalconization was found by Beiler and Martin, in 1951,[7] to be the governing factor for the inhibitory action of the flavonoids against xanthine oxidase. These authors reported maximum activity with compounds having hydroxyl or methoxyl groupings at the 3′ and 4′ positions. Clark and Geissman, in 1949,[35] found that compounds of a flavonoid structure inhibited epinephrine oxidation by virtue of their ability to form metal chelates.

Despite these examples of actions on specific enzyme systems, the main interest in the flavonoids stems from their effects on capillary permeability and fragility. Historically, the prominence of this field of investigation dates from 1936, with the isolation from the peels of citrus fruit by Szent-Györgyi and co-workers[1] of a material which they called "citrin." They showed that this material consisted of a mixture of flavonoids and had the property of reducing capillary fragility and permeability. Bruckner and Szent-Györgyi, in 1936,[16] showed that the active principle in citrin was hesperidin. Since that time, similar activity has been reported for a number of flavonoid compounds, including rutin,[4] esculin, and its aglycone esculetin,[5] and catechin and its epimers.[6] In short, such activity appears to extend to flavones, flavonols, and flavanones as well as to structurally similar compounds, whether combined with carbohydrate or in the aglycone form.

The mechanism by which the flavonoids exert their influence on permeability and capillary fragility has been extensively investigated, and various workers have postulated their interaction with different metabolites and enzyme systems linked to regulation of permeability. The first step in this direction came from Bentsath and Szent-Györgyi,[17] who reported in 1937 that traces of ascorbic acid were required for citrin activity. Since that time, a great deal of evidence

has been adduced to support the idea of a relationship between the flavonoids and ascorbic acid. Gero, in 1947,[9] and Parrot and Cotereau, in 1946,[8] showed that flavonoids protected ascorbic acid against oxidation *in vitro*. The latter authors[10] found that ascorbic acid exercised a similar protective effect on the flavonoids. Bhagvat, in 1946,[11] showed that supplementation of the diet of guinea pigs with hesperidin and ascorbic acid caused a greater increase in growth rate than occurred when ascorbic acid was given alone. Lecoq *et al.*,[12] in the same year, showed that vitamin P, given to guinea pigs on a scorbutogenic diet, retarded the appearance of the symptoms of scurvy and prevented the transient neuromuscular disturbances obtained in rats on a diet free of ascorbic acid. These workers suggested that the hesperidin had a sparing action on ascorbic acid in the animals. While this work indicated that flavonoids could to some extent replace ascorbic acid, other reports showed that the reverse was not true. Gabe and his co-workers, in 1946,[13] found that the changes produced in guinea pigs on a flavonoid-free diet, which were similar to those obtained in scurvy, could not be prevented by ascorbic acid. Parrot and his co-workers, also in 1946, found that the increase in capillary fragility obtained on such a diet was likewise not prevented by ascorbic acid. These reports indicated that the flavonoids could be considered to be specific nutritional factors, and not merely adjuncts to ascorbic acid action.

In 1947, Beiler and Martin[15] postulated that the physiological effects of the flavonoids were due at least in part to an action on hyaluronidase. These workers showed a marked synergistic action of these compounds with ascorbic acid on the enzyme *in vitro*. This is particularly interesting in view of the original observation of Bentsath and Szent-Györgyi that ascorbic acid was required for flavonoid action in the body.

Martin and his co-workers[19] found that the synergism between the flavonoids and ascorbic acid could also be demonstrated in the body. Using the technique of Ambrose and DeEds,[18] they found that the inhibitory effect of hesperidin methyl chalcone on experimentally-induced increase in capillary permeability was markedly potentiated by addition of ascorbic acid. The same synergistic effect was obtained on inhibition of intracutaneous dye spreading, thus providing a direct link with hyaluronidase action in the body.

In view of the effects of epinephrine on vascular permeability, and the fact that polyphenols are known to act as epinephrine antoxidants,[32] it is not sur-

Table 1

Inhibitory Action of Vitamin P Compounds on Hyaluronidase

Compound	0.1 mg./cc.	1 mg./cc.	*Inhibition (per cent)* 0.1 mg./cc. + ascorbic acid 0.1 mg./cc.
Ascorbic Acid	25		
Dicoumarol	35		
Hesperidin	0	0	0
Hesperidin (purified)	0	0	40
Hesperidin methyl chalcone	0	0	75

prising that some workers have attempted to explain the effects of the flavonoids on permeability and fragility by postulating a potentiation of epinephrine action. Lavollay, in 1944,[33] and Parrot and Galmiche, in 1945,[34] reported that certain of the flavonoids which were characterized by the presence of free hydroxyl groupings had the property of inhibiting oxidation of epinephrine. Lavollay correlated this property with the protective effect exerted by these flavonoids against the development of capillary fragility. Clark and Geissman, in 1949,[35] found that the ability of the flavonoids to inhibit epinephrine oxidation could be correlated with the presence in the molecule of a 3′,4′-dihydroxy or 3-hydroxy-4-keto grouping, and explained the action as being due to chelation of copper. These investigators, however, reported that ability to inhibit epinephrine oxidation could not be correlated with capillary activity in the body. The question of the role of this mechanism in the physiological action of the flavonoid compounds therefore remains unsettled. It is of interest to point out that Rayle and Papageorge[36] demonstrated synergism in this system between vitamin P compounds and ascorbic acid.

I suppose that any discussion of permeability might be considered incomplete without reference to histamine at some point. It is true that there is evidence in the literature linking the action of the flavonoids to histamine. Parrot and Richet, in 1945,[20] showed that scorbutogenic diets enhanced histamine toxicity in guinea pigs, and that this enhancement was prevented by catechin epimers. Torii, in 1943,[21] had shown that a soluble hesperidin compound would reduce the toxicity of histamine in normal animals. Wilson and his co-workers, in 1947,[22] reported the same effect with rutin, although this finding was not confirmed by Raiman and co-workers[23] in the same year.

A number of the flavonoid compounds have been found to prevent anaphylactic shock. Antianaphylactic effects were reported for soluble hesperidin derivatives by Hiramatsu in 1941[24] and by Fujihara[25] in 1942; for rutin, by Raiman and his co-workers[23] in 1947; for catechin, by Moss, Beiler and Martin in 1950;[30] and for quercetin, by Thiele and Schuchardt[31] in 1952. Several authors have failed to confirm these results, *e.g.* Roth and Sheppard,[27] in 1948, and Arbesman and Meter,[26] in 1949. It is quite possible that these negative results were a function of the experimental conditions employed, since Martin and his group found that pretreatment of the animals with flavonoid for a considerable period of time was necessary to obtain the antianaphylactic action.

The mechanism for this antianaphylactic action has received much attention. It does not appear to be a direct antihistaminic action; Fujihara,[25] in 1942, showed that it was not elicited against histamine action on isolated tissue, and Moss, Beiler and Martin,[30] in 1950, showed that an animal protected against anaphylactic shock by catechin was not protected against histamine. Ungar, in 1945,[28] reported that flavonoid compounds, particularly hesperidin methylchalcone and epimerized D-catechin, inhibit release of histamine from blood cells. He related effects on capillary fragility to this phenomenon. Martin and his co-workers,[29] in 1949, found that flavonoid compounds were effective inhibitors of histidine decarboxylase *in vitro*, and postulated that they exerted their antianaphylactic effect by preventing the formation of histamine through

Table 2

(1) Direct effect on the capillaries
(2) Potentiation of action of ascorbic acid
(3) Inhibition of hyaluronidase
(4) Inhibition of histamine
    (a) Direct antihistaminic action
    (b) Inhibition of release of histamine
    (c) Inhibition of formation of histamine
(5) Inhibition of epinephrine oxidation
(6) Action on bleeding and coagulation time

inhibition of this enzyme. Ascorbic acid was found to be synergistic with the flavonoids in this system.

Ungar, in connection with his work on histamine release, to which I have just referred, reported that one manifestation of this effect of the flavonoids was a decrease in bleeding time in guinea pigs. A similar report came from Parrot and Galmiche,[37] in 1945; this work was not confirmed by Clark and McKay[38] in 1950.

Finally, although this aspect will be covered in more detail by another speaker, mention should be made of the antidicoumarol effect of the flavonoids, which was reported by Balducci[40] in 1948, and by Martin and Swayne[39] in 1949. The latter workers found that ascorbic acid also counteracted dicoumarol and was synergistic with D-catechin in this respect.

I have attempted to consider briefly some of the physiological actions of the flavonoids, particularly as regards their effects on the vascular system. Table 2 summarizes the explanations which have been advanced to account for these effects. Certainly, at the present time, no single mechanism can be pointed to as being the correct one and, indeed, it would appear that a series of interrelated actions would be the most logical means of accounting for the profound physiological actions of the flavonoids.

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Discussion of the Paper

Doctor Szent-Györgi: Is there any evidence for chelate formation and could synergism with ascorbic acid not be explained by the chelate? Both of them have groups which could join with a metal as a chelate.

Doctor Martin: I believe that the evidence for quinone formation *in vivo* is indirect rather than direct, *i.e.*, rather by correlation of *in vitro* effects with *in vivo* manifestation. In many instances, that correlation has been quite positive. I believe that the aspect of chelation and synergism between the two is an excellent suggestion. I know of no direct work to demonstrate co-chelation by the bioflavonoids and ascorbic acid.

Doctor Szent-Györgyi: Is there any evidence that ascorbic acid with the two hydroxyls has the configuration and double chelate?

Doctor Martin: Not that I know of.

Doctor Manasseh G. Sevag (*Department of Microbiology, University of Pennsylvania, Philadelphia, Pa.*): How does the body dispose of flavonoids?

Doctor Albert N. Booth (*Albany, Calif.*): In some work that we have been doing at the Western Regional Research Laboratory at Albany, Calif., we have isolated no less than three metabolic products from the urine of rabbits and rats given quercetin. First, 3-4-dihydroxyphenylacetic acid; meta-hydroxyphenyl acetic acid and homovanillic acid. This seems good evidence that something like quercetin, the aglycone of rutin, is definitely absorbed. Whether these metabolites in themselves have physiological activity is an unanswered question.

Doctor Rosenthal: We have the answer for the contradictory results with flavones in anaphylactic shock. I should like to recall an experiment I made at the University of Budapest which showed that the reaction was quite often different according to the season. Some experiments carried out in the spring gave different answers from those performed in the summer or fall. This may account for the contradictory answers observed.

DOCTOR MARTIN: This undoubtedly accounts for the variation as season and basic nutritional status would be related.

DOCTOR SEVAG: Does the body manufacture any of these flavonoids, or are they entirely dependent upon outside sources?

DOCTOR MARTIN: I know of no evidence that the body synthesizes any flavonoid molecule. I believe that the future will demonstrate true vitamin characteristics of the bioflavonoids. This automatically carries with it the implication they are not synthesized.

ESTROGENIC ACTIVITY OF SOME NATURALLY OCCURRING ISOFLAVONES*

By Edmund W. Cheng, Lester Yoder, Charles D. Story, and Wise Burroughs

Iowa Agricultural Experiment Station, Ames, Iowa

The biological significance of the naturally occurring isoflavones was not recognized until recently, although several of them had been isolated, chemically characterized, and synthesized. Perhaps the first evidence indicative of the biological activity of isoflavones was the fact that a serious breeding problem in sheep occurred when these animals were grazed on subterranean clover (Bennetts *et al.*, 1946), due to the presence of an estrogen or estrogens. Subsequently, the isoflavone, genistein, was isolated from the subterranean clover (Bradbury and White, 1951) and was considered to be the principal estrogen in that plant (Biggers and Curnow, 1954).

Our interest in the estrogenic isoflavones was initiated three years ago when it was observed that the growth stimulating effect of stilbestrol implantation noted by others with lambs was not obtained (Culbertson, 1952) when the lambs received liberal amounts of alfalfa and clover hays. Attempts were then made to determine whether the hays used in this experiment contained any estrogenic activity. This work eventually led to a general survey of estrogenic substances in feeds, including soybean oil meal, which was known to contain considerable amounts of an isoflavone glucoside, genistin (Walz, 1931; Walter, 1941). Apparently isoflavones are responsible for at least a part, if not all, of the estrogenic activity that has been found in many plant products. Subsequently, some of the common naturally occurring isoflavones were synthesized in our laboratory and their estrogenic activity was determined. This report will treat some of these experiments in detail.

Experimental

Estrogenic activity of hay extracts. The procedures for the extraction of hays were described by Cheng *et al.* (1953a). Immature female mice from 8 to 10 gm. in weight were used for the biological assay of estrogenic activity. The extract was injected subcutaneously at a daily dose of 0.05 ml. per mouse for four days. The animals were killed 24 hours after the last injection. The uteri were dissected out and fixed in Bouin's fluid for 24 hours. They were then dried by pressing against filter papers and weighed. Among the extracts assayed, those of clover and alfalfa hays showed some estrogenic activity as shown in TABLE 1.

When the two first cutting hays are compared, there is little difference in the estrogen content, but when the second cutting hays are compared it is shown by TABLE 1 that alfalfa hay contains less estrogenic activity (1.61 μg. stilbestrol per pound of hay) than that found in the clover (1.95 μg./lb.). It is interesting to note that second-cutting clover hay contained more estrogenic activity than

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TABLE 1

BIOLOGICAL ASSAY OF ESTROGENIC SUBSTANCES IN HAY EXTRACTS

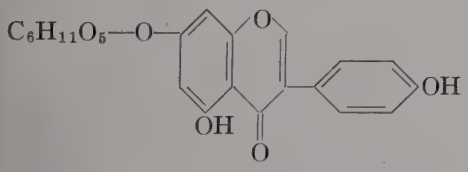
Extracts	Dose*	No. of mice	Uterine wt. mg. average	Estimated potency in μg. stilbestrol per lb. of hay
Control		20	7.4 ± 2.3	—
First-cutting clover	5 g.	6	15.1 ± 2.6	0.87
Second-cutting clover	5 g.	9	32.4 ± 5.9	1.95
First-cutting alfalfa	5 g.	9	15.5 ± 4.9	0.88
Second-cutting alfalfa	5 g.	6	26.4 ± 4.3	1.61

* Weight of air-dry materials represented by dose of extract administered daily.

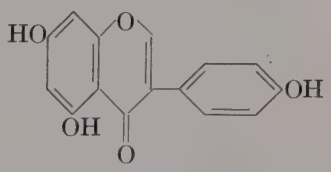
first-cutting clover hay. Also, the same relationship existed in two alfalfa hays tested, namely, second-cutting alfalfa was more potent in estrogen content than the first-cutting sample. Whether any significance can be attached to this relationship is not known at present. Possibly the estrogen content increased with the maturity of the plant. Legg *et al.* (1950) found that red clover cut in February had less activity than that cut in March, but the potency declined in June. Samples of blue grass, brome grass, ladino clover, and the immature wheat plant assayed in this study failed to show any estrogenic activity.

In view of the rich occurrence of isoflavones in some plants, it is possible that some of the isoflavones may be responsible for the estrogenic activity, but the specific substance responsible has not been definitely identified to date.

Estrogenic activity of isoflavones in soybean oil meal. The presence of an isoflavone glucoside, genistin, in soybean oil meal has been known for some time. Since soybean oil meal is used rather widely in livestock feeding, it appeared advisable to investigate the isoflavones contained therein and to determine their estrogenic activity. Accordingly, commercial soybean oil meal (solvent process) was extracted with methanol according to the method of Walter (1941).



(I)



(II)

Genistin (I) was isolated as pale yellow, thin rectangular plates having a melting point of 256° C. in an amount of about 0.1 per cent. This compound is a glucoside of genistein (II), 5,7,4′-trihydroxyisoflavone. Genistein was obtained by hydrolysis of genistin with hydrochloric acid in methanol. It was crystallized from hot 60 per cent ethanol as white rectangular rods having a melting point of 298° C.

The estrogenic activity of these compounds was determined by the mouse uterine weight method. The chemicals under study were either mixed in the ration and fed directly to the immature female mice or injected subcutaneously as their sodium salts. The results are shown in TABLE 2 (Cheng *et al.*, 1953b).

Feeding 2.5 and 5.0 mg. of either genistin or genistein per day per mouse resulted in increased uterine weights. Injecting genistein at 1 and 2 mg. levels also increased uterine weights consistently over the corresponding weights of control animals. Whereas the injection of 1 mg. of genistin did not have a measurable effect, the injection of 2 mg. proved quite effective. It should be noted that these responses are similar to those due to the injection of 0.02 to 0.04 μg. of diethylstilbestrol as shown in TABLE 2. The estrogenic activity of genistein can accordingly be estimated as approximately equivalent to 1/50,000 the activity of diethylstilbestrol. Genistin activity on a weight basis was slightly lower than that of genistein. The two compounds, however, appeared to have approximately equal activity on a molecular basis.

Estrogenic activity of some synthetic isoflavones. Since there are several known isoflavones present in natural plant material, it seemed worthwhile to determine which of these compounds are estrogenic. Our next step was to synthesize, chemically, some of these naturally occurring isoflavones. The reactions involved in the synthesis of genistein are illustrated as follows:

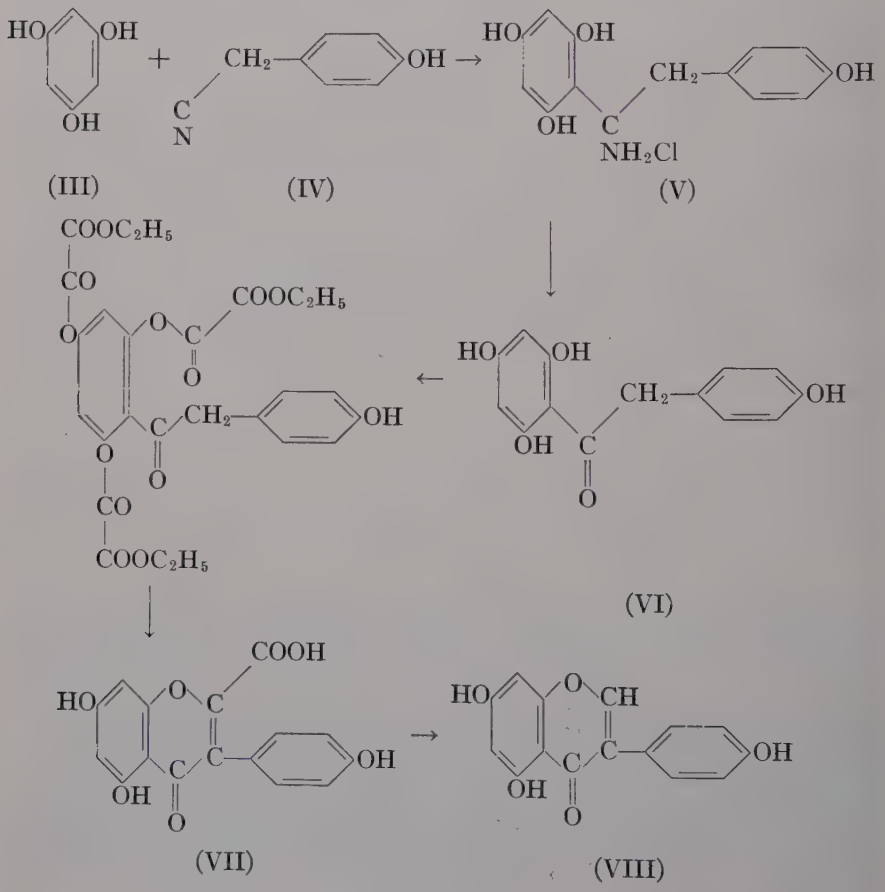


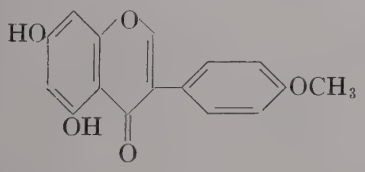
Table 2
Estrogenic Activity of Genistin and Genistein

No. of mice	Treatment*	Average uterine weight mg.
6	Normal control	9.7 ± 2.8
6	Feeding genistin, 2.5 mg.	12.9 ± 4.4
6	Feeding genistin, 5.0 mg.	39.8 ± 8.9
6	Injecting genistin, 1 mg.	9.2 ± 1.7
6	Injecting genistin, 2 mg.	14.6 ± 3.6
6	Feeding genistein, 2.5 mg.	21.6 ± 13.4
6	Feeding genistein, 5.0 mg.	22.6 ± 4.6
6	Injecting genistein, 1 mg.	13.2 ± 2.4
6	Injecting genistein, 2 mg.	17.0 ± 7.3
6	Injecting stilbestrol, 0.02 μg.	13.2 ± 2.6
5	Injecting stilbestrol, 0.04 μg.	18.3 ± 6.9

* Treatment was given daily for four days.

Ketimine hydrochloride (V) was prepared by the condensation of phloroglucinol or resorcinol (III) with p-hydroxyphenylacetonitrile (IV). Upon acid hydrolysis of ketimine hydrochloride (V), deoxybenzoin (VI) was obtained. The formation of the pyrone ring of the isoflavone was accomplished by reacting the deoxybenzoin (VI) with ethyl oxalylchloride in pyridine. Upon saponification the 2-carboxyl derivative (VII) was obtained. Pyrolysis of this compound at the melting points evolved carbon dioxide to yield the isoflavone (VIII) desired. The details of the synthetic processes are published elsewhere (Yoder *et al.*, 1954).

Besides genistein, three isoflavone derivatives have been synthesized. These are biochanin A (IX), daidzein (X), and formononetin (XI).



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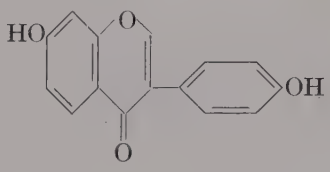

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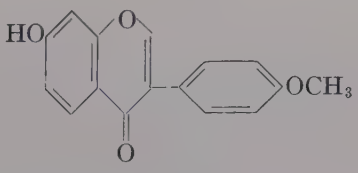


(XI)

Unpublished data in this laboratory indicated that both synthetic and naturally occurring genistein have equal estrogenic activity. Consequently, only synthetic isoflavone compounds were tested. These compounds were fed to mice at a level of 1.25 mg./g. of ration in assaying their estrogenic activity by means of the uterine response technique. The respective isoflavone compounds were first dissolved in ethanol, then mixed with the basal ration, and the

TABLE 3
ESTROGENIC ACTIVITY OF SOME ISOFLAVONE DERIVATIVES

No. of mice	Treatment	Average uterine weight (mg.)	Approx. potency*
6	Normal control	6.4 ± 0.8†	
6	2.5 mg. biochanin A	20.9 ± 3.1	0.033
5	2.5 mg. daidzein	26.6 ± 4.1	0.042
6	2.5 mg. formononetin	8.9 ± 1.2	0.009
5	2.5 mg. genistein	19.3 ± 1.3	0.030
6	0.01 μg stilbestrol	9.4 ± 0.8	
6	0.02 μg stilbestrol	15.7 ± 1.5	
6	0.04 μg stilbestrol	22.2 ± 2.1	
5	0.08 μg stilbestrol	46.2 ± 4.7	

* Expressed as micrograms of diethylstilbestrol activity.
† Standard deviation.

ethanol evaporated from the completely mixed ration. Since the mice consumed an average of 2 g. of diet daily, the average intake of the respective compounds was 2.5 mg. daily over the experimental period of 4 days. The results of this experiment are presented in TABLE 3 (Cheng *et al.*, 1954).

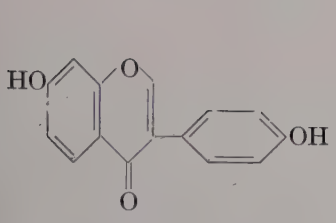
It is readily apparent that each of these isoflavones is estrogenic in nature. Daidzein appears to be the most active, and genistein and biochanin A have approximately equal activity, whereas formononetin shows the least estrogenic activity. From the structural formula, it can be seen that formononetin is the only compound tested that has only one free hydroxyl group. Since the activity of many synthetic estrogenic compounds is known to be related to the number and arrangement of hydroxyl groups (Solmssen, 1945), it is not surprising that formononetin proved to be less active than the other compounds tested.

Biochanin A was recently isolated from red clover and found to be estrogenic (Pope *et al.*, 1953). The isoflavone daidzein has not been reported as being present in nature; however, its glycoside, daidzin, has been isolated from soybean oil meal (Walz, 1931).

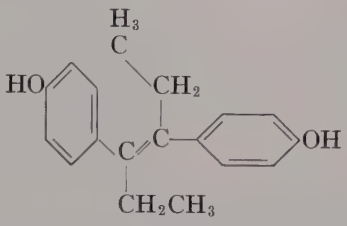
Discussion

Among the 11 known simple naturally occurring isoflavones listed by Warburton (1954), four of them were shown by work in our laboratory to be estrogenic. It will not be surprising, as the search continues, that other isoflavones will be found to be estrogenic. Due to the very low potency of these compounds, it may be difficult for isoflavones to account for all of the estrogenic activity of plant extracts, especially in the case of subterranean clover (Biggers and Curnow, 1954). It is possible that some biological transformation of these compounds may occur with the production of more active intermediates. In view of the fact that daidzein (X) is estrogenic, it may be of interest to compare its structural formula with that of the synthetic estrogen, diethylstilbestrol (XII), which is approximately 50,000 times as potent as any of the estrogenic isoflavones.

The structural similarity of these two types of compounds could be inter-



(X)



(XII)

preted to suggest that isoflavones might be the precursors of estrogens. Until more information about the metabolism of the isoflavones is known, however, isoflavones *per se* probably should be considered as a class of naturally occurring estrogens of plant origin.

Very little is known, at present, concerning the roles these isoflavones might play in plants and animals. Work by Carter *et al.* (1954) indicated that when genistin was included in the ration of mice at a level of 0.2 per cent, there was no apparent effect on growth and reproductive performance. Recent development of the incorporation of diethylstilbestrol in cattle rations for the stimulation of rate of gain in growing and fattening steers (Burroughs *et al.*, 1954) obviously gives added interest and importance to these isoflavones. When one considers that a large amount of isoflavone-rich feeds, such as legume hays and soybean oil meal, is consumed by farm animals, sufficient estrogenic substances may be present in these feeds to exert an important though often nonrecognizable influence upon their physiological functions.

Summary

Estrogenic substances have been extracted from clover and alfalfa hays. An estrogenic isoflavone glucoside, genistin, has been isolated from soybean oil meal. Four isoflavones, genistein, formononetin, daidzein, and biochanin A, have been synthesized chemically and their estrogenic activity assayed by use of the mouse uterine weight method.

The possible structural relationships between isoflavones and diethylstilbestrol and the importance of estrogenic isoflavones in livestock feeds are discussed.

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Discussion of the Paper

Doctor Sevag: Can you give me some proof regarding the exact structure of biochanin A?

Doctor Cheng: The structural formulas of biochanin A seems to be pretty well established because recently the British workers isolated it from red clover hay, using chromatography for isolating this compound, and confirmed its chemical structure.

Doctor Sevag: What is the increase in weight *in utero*? Is it fat, protein, or carbohydrate? If it is fat, is there any possible relationship between it and the increase of the fat weight of the lamb to which you fed soybean, that is, as a consequence of feeding or injecting genestin or other estrogenic substances.

Doctor Cheng: The increase of uterine weight, I believe, is a physiological effect of the female sex hormone. It stimulates the uterus so that it enlarges in preparation for its sexual function. I do not know whether this increase in weight is due to any particular substance, but I have the impression that it was caused mostly by increase of water content and consequent enlargement of the uterus.

Doctor Sevag: Is the increase based on dry weight determination?

Doctor Cheng: The actual procedure that we employed for this estrogenic assay was to fix the uterus in Bouin's fluid. This hardens the tissue and, after 24 hours or so, we take it out, press it between filter papers, and weigh it on the torsion balance.

Doctor Harold Blumberg (*Endo Products, Inc., Long Island City, N.Y.*): Is this estrogenic activity exhibited also by the bioflavonoids in general, such as those mentioned by Doctor Martin in his paper?

Doctor Cheng: The flavones have never been reported to have any estrogenic activity. The isoflavones were known for a long time, but these compounds were never tested until recently, when, due to Australian work, we became interested in the problem.

Doctor Ernest Q. King (*Food and Drug Administration, Department of Health, Education, and Welfare, Washington, D. C.):* Did you run any toxicity experiments? What would be the safety margin, *i.e.* the ratio between the estrogenic activity and the toxicity?

Doctor Cheng: The highest levels of isoflavone that we fed to the mice were not very large, but much larger than other active material. If one feeds very large amounts of the compounds of any type, they would be toxic. As yet we do not have the established level of toxicity.

Doctor King: What would be the potential ratio to estradiol?

Doctor Cheng: This isoflavone group of compounds is active, and their potency is about 1/50,000 that of stilbestrol. The potency of stilbestrol and the natural female sex hormone estradiol were reported in various laboratories to

differ in value. My impression is that the diethyl stilbestrol is about twice as active as estradiol.

Doctor King: In this regression line that you plotted, did you plot the dose or the logarithm of the dose?

Doctor Cheng: The curve is plotted from the dose, not the log-dose. We have also plotted the log-dose on those curves and it did not look to us that it would show any better.

Doctor King: Are the slopes quite similar when you plot the logarithm?

Doctor Cheng: Yes, but the slopes were different when a number of dose-levels were used.

Doctor Szent-Györgyi: Doctor Cheng, you mentioned sterility in the beginning of your paper. Is this related to estrogenic activity?

Doctor Cheng: When the estrogens are used in such high concentration as is present in subterranean clover in Australia, they induce sterility. At our State College of Agriculture and Mechanic Arts, at Ames, Iowa, we found that when estrogenic substances were fed to cattle in small amounts they actually stimulated the growth of cattle about 20 per cent. Quite recently, it has been recommended that diethylstilbestrol be incorporated in feed supplements for cattle. This is the most recent development that we have in the agricultural field. We did not conduct sterility tests but in the literature we found that in Australia, when the lambs got a lot of subterranean clover, which contains about 10 micrograms of diethylstilbestrol activity per pound of hay, they failed to reproduce. In other words, many of the sheep were made infertile. This result was due to the excessive intake of estrogens.

BIOGENESIS OF THE FLAVONOIDS

By Franz Moewus
New York, N. Y.

Botanists are used to thinking in terms of life cycles. An individual life starts as a fertilized egg or zygote. This zygote gives rise to a new organism, more or less morphologically differentiated, which grows or exists for a certain time. At a certain age or at certain intervals this organism undergoes physiological changes, which result in the production of reproductive cells or gametes. The characteristic feature of a gamete is its tendency to fuse with a second gamete in order to reestablish a new zygote.

The reproductive part of the life cycle is characterized by the appearance of special metabolites, *e.g.*, pigments or scents. Although it is a general opinion that these metabolites are produced by the plants in order to make the final fusion of a female and a male gamete possible, there is not yet sufficient experimental evidence to verify this suggestion. This means that we still do not know whether the flower pigments have a biological function in the fertilization process.

We have studied sexuality and fertilization in the most primitive green plant which exists, the unicellular, biflagellated alga *Chlamydomonas eugametos*. It has two mating types which are morphologically alike. This alga can be maintained permanently in the growth phase of its life cycle by subculturing, but it can be transferred into the sexual state of life under certain culture conditions. This switchover from a growing cell to a gamete is not connected with any morphological changes or cell divisions. In our species, we can turn the whole population of an agar plate into a population of gametes within 15 minutes if we use special standard methods. If suspensions of gametes of both mating types are combined, we get an immediate reaction. Gametes clump and, after a few seconds, we see the first pairs. This pronounced copulation reaction is the basic bioassay for all our investigations.

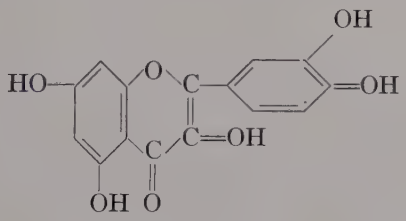
We found that, during these 15 minutes, two kinds of pigments are involved in the sexual process: carotenoids and flavonoids.[1] Carotenoids are secreted and are responsible for the chemotactical attraction of the gametes. It could be shown that this secretion does not occur unless, in a previous phase, another pigment is produced within the gamete. In this prephase, the flavonoid, isorhamnetin, could be shown to be formed in female gametes, while male gametes produce the anthocyanin, peonin. Both pigments could be isolated in crystallized form from gamete material by Kuhn and Löw.[2] Besides, we had at our disposal a sterile *Chlamydomonas* strain which would not copulate under standard conditions and, from this strain, Kuhn and Löw isolated rutin.[3] We can understand the sterile behavior of this strain if we assume that rutin interferes with the secretion of the carotenoid chemotactic agents.

By mutation experiments which were carried out from 1939 to 1951 we obtained a number of mutants from the wild type which did not show the normal behavior in the copulation test. These were female and male sterile mutants.

Sterility in the females could be overcome if isorhamnetin were present during the short phase of induction of sexuality, and the same thing is true with peonin in the males. This means that the sterility is due to the inability to form the two pigments. We also obtained mutants from the sterile, rutin-forming strain, which turned out to be fertile. That means the production of rutin was blocked. Thus, the sexual process was no longer disturbed. From this fertile mutant, Kuhn and Löw[2] were able to isolate another flavonoid, quercetin. Quercetin seems to be a precursor of rutin but, since this mutant has normal sexuality, we concluded that quercetin may be also the precursor of the two other pigments, isorhamnetin and peonin. Quercetin seemed to have a central position in the biogenetical pathway which normally leads to isorhamnetin and peonin, and has a side branch to rutin in the sterile strain.

We proved this by testing 37 sterile female mutants, which all became sexual in the presence of isorhamnetin, but only 26 could be made sexual by quercetin. Eleven sterile mutants were obviously not able to synthesize the final product from the offered quercetin. Accordingly, quercetin must be a link in the synthetic pathway.

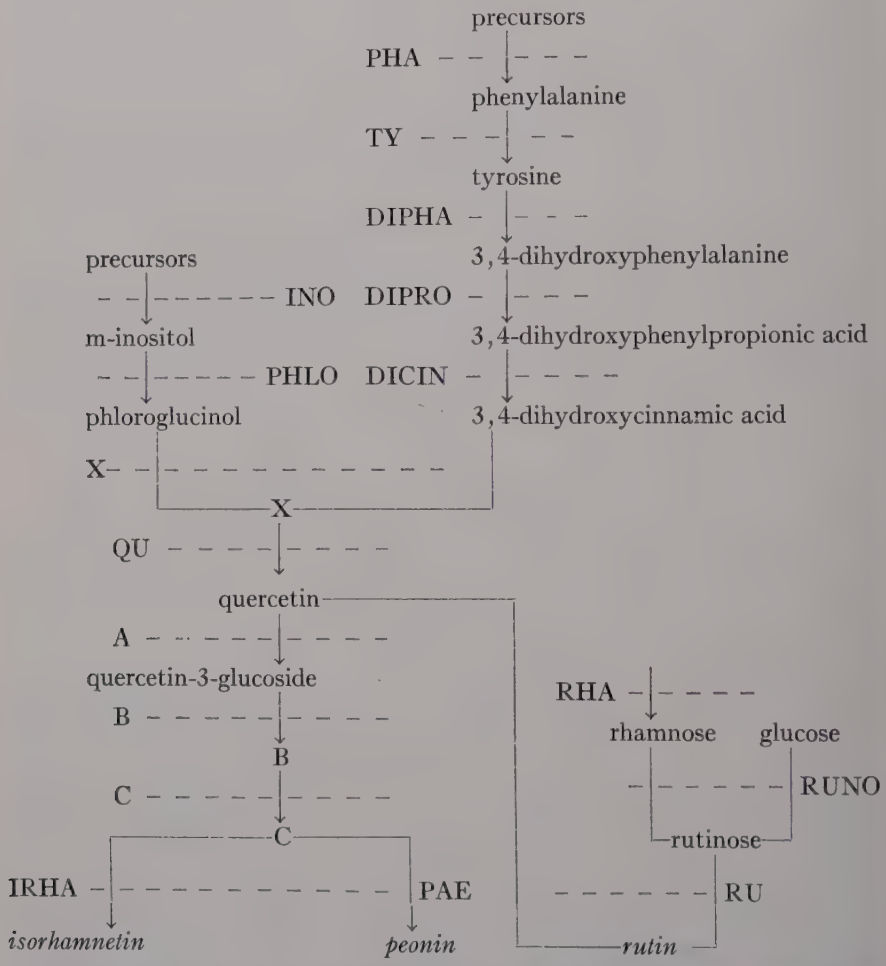
Quercetin is very common in higher plants, and it seemed to be promising to trace back its biosynthesis, using our unicellular green alga. The quercetin molecule consists of two C_6-units which are connected by a C_3-chain:



To investigate the biosynthesis of this molecule, simpler compounds suspected to be its precursors had to be offered to the above-mentioned 26 mutants. I want to make it quite clear: the substances are not given during the growth period on agar. They are offered only during the period of sex determination The cells grow on normal mineral salt agar. Then they are placed for four hours in a solution of the substance to be tested while sex determination can take place. Now we do the copulation test, adding male gametes. The answer is either copulation or no copulation. Copulation indicates that the provided substance is a link in the chain of the quercetin synthesis, and that the sterility has been overcome.

For these substitution tests, about 30 compounds were available. Among these only eight gave a positive copulation test: meso-inositol, phloroglucinol, d-, d,l-, l-phenylalanine, l-, d,l- tyrosine, 3,4-dihydroxyphenylalanine, 3,4-dihydroxyphenylpropionic acid, 3,4-dihydroxycinnamic acid, and quercetin. Inactive were, *e.g.*, 3- and 4-hydroxycinnamic acid, 4-hydroxy-3-methoxycinnamic acid, cis- and trans- cinnamic acid, phenylpropionic acid, 3- and 4-hydroxyphenylpyruvic acid, phenylpyruvic acid, phenyllactic acid, 3,4-dihydroxybenzoic acid, methylphloroglucinol, epi-inositol, and pyruvic acid.

From the chemical viewpoint, it seems to be impossible to arrange the eight active compounds in a linear sequence. On the one hand, inositol and phloroglucinol are closely related. On the other hand, the compounds from phenylalanine to 3,4-dihydroxycinnamic acid seem to form a linear sequence. Therefore the following scheme of the biogenesis is indicated:



The assumed scheme was supported by further biological experiments. We were able to show that, at each genetical block, the previous product was accumulated and secreted. For instance, the PHLO-mutant, which cannot produce phloroglucinol, secretes inositol. If we separate the PHLO-cells from the medium by centrifuging, we obtain a supernatant which contains inositol. This inositol preparation can be used for activating the INO-mutant, for INO is not able to synthesize inositol. By this method, we have shown that the PHLO-mutant produces inositol, and that the INO-mutant is next to the

PHLO-mutant. Using this centrifugate method, we could place the five phenyl-derivatives in the mentioned order.

We have good reason to believe that the two independent sequences are linked together and form, finally, the quercetin molecule. Namely, we have the mutant X, in whose supernatant we found phloroglucinol and 3,4-dihydroxycinnamic acid, as well. Obviously this mutant cannot perform the linkage of the two branches. The X-substance is expected to be the first flavonoid with the typical $C_6—C_3—C_6$ structure. We suspect it to be luteolin. From luteolin to quercetin only one methylation has to be carried out by the cell, which may be regulated by one gene. Thus, X is probably the last precursor of quercetin.

The biological part of this investigation was supported by chemical identification of quercetin and its precursors from alga material. This work was carried out by Birch, Donovan and Moewus.[4] Using standard methods of paper chromatography, they could identify inositol from the PHLO-mutant, phenylalanine from the TY-mutant, tyrosine from the DIPHA-mutant, 3,4-dihydroxyphenylalanine from the DIPRO-mutant, 3,4-dihydrophenylpropionic acid from the DICIN-mutant. The X-mutant material contained, indeed, phloroglucinol and 3,4-dihydroxycinnamic acid as well.

Just recently, Geissman and Harbone[5] reported the occurrence of cinnamic acid derivatives in a white flowering mutant of *Antirrhinum*, while the normal wild type is pigmented by flavonoids. Thus, he got the same blockage at the place of our X- or QU-mutant. Moreover, he found in three other plants that the flowers contain 3,4-dihydroxycinnamic acid esters and flavonoid pigments hydroxylated in 3,4-position. Also, peach leaves contain kaempferol and quercetin along with esters of both 4-hydroxycinnamic and 3,4-dihydroxycinnamic acid. This part of our biosynthesis seems to be a rather common pattern in green plants.

Summing up the experimental results, we can draw the following conclusions:

(1) Since none of the genetical blocks in our mutants has a lethal effect, we are not dealing with a life-important biosynthesis. We have to think of a secondary biochemical process which is carried out at a very low molecular level during the growth process.

(2) We must assume that our final products are channeled off by other biochemical reactions. Otherwise, we could not explain why the intermediates are accumulated in mutants.

(3) If growth processes have slowed down and conditions for sexuality are realized, the secretion of the final products is set in motion and, in this way, the secondary process becomes predominant now. Thus, the level of production of our pigments is raised now within the level of experimental detection. The biological tests are more sensitive. That is why we can trace isorhamnetin and peonin in filtrates of the wild type. For chemical isolation, however, we must use genetical blocks in order to raise again the level of production. Otherwise, we should have to grow kilograms of algal material.

(4) Although it seems that the three mentioned flavonoids, isorhamnetin, peonin, and rutin, have obviously a very specific function in the sexual process

of our unicellular alga, presumably regulating the output of the carotenoid chemotactic agents, there is some reason to believe that they are involved in other nonspecific functions in this microorganism and in other plants as well.

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Discussion of the Paper

Doctor Szent-Györgyi: Is there any difference in the reaction between the male and female gametes?

Doctor Moewus: No. They have all the same genes, except as to the flavonoid system, the gene irha in females, the gene pae in males.

Doctor Marjorie B. Zucker (*New York University College of Dentistry, New York, N. Y.*): What causes the change from the nonsexual to the sexual form of the algae?

Doctor Moewus: This question is very difficult to answer. If one cultivates the alga material on agar, the cells are in the vegetative phase. If flooded with water under certain light conditions, where the proportion of the red wave lengths and the blue wave lengths have a certain value, one gets the induction of sexuality.

Doctor Sevag: In view of the work of Mitchell in California, and also of Werner Davis, some of these biological blocks or genetic blocks are not actually blocks but the accumulation of certain compounds which act as inhibitors and prevent the next step. Is there some such mechanism involved in your mutants? The way to get around that is to use an antagonist of the accumulated substance to relieve the inhibition and allow the cell to proceed to the next step.

Doctor Moewus: I think that is possible, but we have not done any experiments on this problem. In any case, this biosynthesis cannot be primarily essential to life, because otherwise we could not cultivate our cell material on mineral salt agar. A mutant would die if it were a primary synthesis. That is the problem we are struggling with at the moment.

Doctor J. Munoz (*Sharp & Dohme, Philadelphia, Pa.*): How much of this do you require to produce the changes and in what form do you add the quercetin?

Doctor Moewus: We find that 10^{-8} to 10^{-9} gram quercetin per milliliter are necessary, and we make, first, an alcoholic solution and then dilute it.

ANATOMICAL AND FUNCTIONAL CHANGE IN THE PERIPHERAL VASCULAR SYSTEM DURING CERTAIN INDUCED INCREASES IN VASCULAR FRAGILITY*

By Richard E. Lee, David Goebel, and Lyman A. Fulton
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Our study of nutritional elements and peripheral vascular physiology began some years ago with the development of techniques for study of the capillary bed in mammals.[1] These could be applied to evaluating increased vascular fragility as a result of scurvy and of other food deficiencies in the intact living animal. It seemed of interest, where histological and cytological studies failed to reveal outstanding changes in the vascular system *per se*, to study the living functional capillary bed during avitaminosis C.[2]

Living unanesthetized guinea pigs were trained to lie in a special holder beneath the microscope. Through a procaine fieldblock in the flank the peritoneal cavity was entered and the viscera allowed to extrude into a warm 1 per cent gelatin saline bath, continually washed by fresh lavage fluids. The mesenteric areas were then searched for a suitable vascular area to be studied under the microscope at magnifications of 50 to 200 power. The velocity of blood flow through arterioles, capillaries, and venules was estimated; the reactivity of the terminal arterioles and precapillary sphincters to direct application of epinephrine in known concentrations was measured; the diameters of the vessels were measured with an ocular micrometer; and, finally, the reaction of the vessels to direct trauma (brushing with a camel's hair brush) was determined in each instance. These studies were repeated on control animals supplemented with 15 mg. of ascorbic acid daily per 100 grams of body weight, and on animals completely deficient in vitamin C. The period of deficiency was from 18 to 20 days. At the end of this time, the tissue concentration of ascorbic acid had fallen to a level of less than 5 per cent of the normal. On our synthetic diet, the animals routinely succumbed within 26 to 34 days. Just prior to death, they invariably exhibited the classical laboratory symptoms of scurvy.

Deficiency in vitamin C was found to be associated with dilatation of all vessels and reduced velocity of blood flow throughout the capillary bed. In addition, there was decreased responsiveness in the muscular arterioles and precapillary sphincters to the direct application of epinephrine.[2] As the animals were conscious, the controls frequently reacted rather violently to environmental stimuli. For example, the slamming of a door would cause them to become startled and, subsequently, to develop a profound vasoconstriction in the area observed in the mesentery. While this was most notable in all control animals, the animals deficient in vitamin C were characterized by an almost complete absence of this splanchnic vasomotor response to fright.[3] Trauma to the vessels was accompanied by no significant petechiae formation in the control animals. The deficient animals, on the other hand, demonstrated vascular ruptures in almost every instance. Ninety per cent of the hemorrhages

* This study is supported by the Nutrition Foundation, New York, N. Y., and the A. H. Robins Foundation for the Study of the Degenerative Diseases in Man, A. H. Robins Co., Richmond, Va.

were from the venules. The increased venular fragility elicited by trauma in vitamin C deficiency, as measured, was correlated with no increase in arteriolar or capillary fragility *per se.*

The trauma resulted in a direct tear of the vessel wall, or it perhaps produced irritation of the wall and an increased porosity with escape of formed blood elements. In most instances it was possible to see that the wall of the venule had a usually "tricornered" tear, through which the cellular elements of the blood were observed to escape. This was by no means the invariable rule, however, for in and around many of the petechiae no such vascular rupture could be seen. Whether it was on the other side of the venule or whether the extravasation of blood took place through stretched vascular pores could not be determined in these instances.

Vascular Fragility in Hamsters

In studies concerning the influence of various metallic cations in the diet on the peripheral vascular system, it was discovered that the feeding of excess sodium to the Syrian hamster was often accompanied by numerous petechiae in the capillary bed of the cheek pouch.[4] These formations appeared within 10 to 30 minutes after preparing the vessels in the pouch for observation with a microscope. It was of interest to note that, as with the scorbutic guinea pigs, these petechiae in the cheek pouch of the hamster appeared in association with the collecting venule system. In all animals, it was possible to observe the venules before petechiae were present and to note the gradual loss of blood into the tissues at specific sites along the venular tree. There was no possibility that these cells were extravasated elsewhere in the vascular system and then migrated along the capillaries to appear as petechiae at the venule. The cause of petechiae formation under these circumstances is not clear. A few studies to date on plasma levels of sodium indicate that it may reach considerably elevated levels in sodium-fed hamsters. It is conceivable that this may make the usual perfusion medium relatively hypotonic and produce cellular swelling with rupture of the vascular wall.

Acute Choline Deficiency in Rats

The frequently widespread hemorrhagic manifestations of young choline-deficient rats have been clearly established. The question naturally arose regarding the nature of the peripheral vascular derangement of this dietary deficiency, so predisposing to a bleeding tendency. Forty young albino rats of from 21 to 30 days old were therefore placed on a choline-free high fat (30 per cent) diet for seven to nine days. At the end of this period, the mesoappendix was examined microscopically with the animal under pentobarbital anesthesia. There was a significant reduction in the number of patent arterioles per unit of tissue, and arterioles and precapillary sphincters were notably hyperreactive to epinephrine. Particularly prominent was a tendency of the vessels to rupture at direct mechanical trauma, and at milliseconds voltages of electrical stimulation with micromanipulation that customarily produced no observed vascular damage. Of interest was the fact that this vascular fragility occurred

in over 90 per cent of instances in the collecting venules,[5] in vessels of a calibre similar to those rupturing in the other experimental nutritional conditions described above.

Studies in the Capillary Bed of Man

In our survey work with regard to the peripheral vascular system in the bulbar-conjunctiva of normal and diseased individuals, we occasionally see spontaneous petechiae present in the capillary bed of our subjects. These formations are seen almost invariably in association with the collecting venular system,[6, 7] with definite anatomical change in the venule. At certain areas, the venules have a fusiform swelling of the wall resembling a "plumber's joint" in a lead pipe, a ballooning outward of the entire vascular wall. From such dilated areas, red cells may be seen to extravasate. They rupture through the venular wall as if under considerable pressure, and may be deposited in sites considerably remote from the dilated "parent" venule.

Comment

The observations indicated above for scorbutic guinea pigs point up the fact that increased venular fragility is prominent. It is accompanied by arteriolar and venular dilatation with reduced peripheral flow of velocity, and a diminished vascular reactivity to stimulation with epinephrine. In choline-deficient rats, however, the increased venular fragility is associated with arteriolar narrowing, with a sparsity of arterioles and patent capillaries in the field, and an augmented vasoconstriction response to epinephrine. The vessels in the cheek pouch of the hamster supplied with 1 per cent saline rather than water show no arteriolar or capillary derangement under the microscope, yet spontaneous petechiae formation may be widespread. It is therefore apparent that in all three species and under various conditions, these may greatly differ in physiological changes in the capillary bed, and yet the phenomenon of increased peripheral vascular fragility is present in each, with petechiae formation uniformly manifested by the loss of red cells from the venules.

In the five human subjects observed to date with spontaneous petechiae formation in the bulbar conjunctiva, the petechiae are seen in close association with venular changes. These latter consist of annular and occasionally saccular widening with reduced venular blood-flow velocity in the widened portion, a turbulance of blood flow, and an observed escape of red cells from the distorted segment.

Increased fragility in the capillary bed is therefore a venular defect. It is associated with a variety of experimental nutritional states, and with differing associated peripheral vascular manifestations in each. Supplying vitamin C to the scorbutic guinea pigs corrects their vascular fragility along with dilatation and hyporeactivity. Choline supplementation to the deficient rats may restore normal fragility and also reduce the vasoconstriction present, as well as the augmented reactivity to drugs. Withdrawal of saline from the hamster prevents the spontaneous petechiae formation in the venules. These data suggest that in order to treat a hemorrhagic diathesis properly, it is necessary to obtain

more knowledge in regard not only to the specific defect in the diet that may be responsible for the bleeding, but also to the associated vascular phenomena that may be playing a role in the bleeding tendency.

It is of considerable interest that the site of blood loss in each above instance was the collecting venule. The true capillaries, where trauma may be followed by precapillary constriction preventing loss of blood, and where the volume of blood in single vessels is much less than one finds in the larger collecting venule, are not the primary site of petechiae formation. It therefore becomes of importance to attempt to extend knowledge of the physiology and the anatomy of the venule and its role in peripheral vascular physiology. These vascular elements have long been considered to be little more than a system of collecting tubular conduits whose function is almost entirely that of returning the blood used by the tissues to the central vascular system for refurbishing and redistribution. They conceivably may have far more important roles in exchange between the intravascular blood and the tissue spaces.

Interests of late have included micromanipulator studies of the peripheral vascular bed during nutritional deficiencies, as well as in normal animals. In the various nutritional states studied to date and in "normal" animals of all three species, micromanipulation and rupture of the various elements in the peripheral vascular bed with direct trauma and with various millisecond volt stimuli through microelectrodes confirm the concept that the venule is the most susceptible to leakage of red cells. It is therefore likely that specific dietary conditions, by inducing differing vascular changes in the capillary bed, will predispose, by various mechanisms, the naturally "weakest" peripheral vascular element to blood loss. Petechiae formation may be chiefly a nonspecific phenomenon resulting but secondarily from other and more specific functional and anatomical vascular derangements.

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Discussion of the Paper

Doctor Douglas E. Johnstone (*University of Rochester Medical School, Rochester, N. Y.*): Would it be possible to use the nail bed which has been used in so-called capillary microscopy as a tool to study this phenomenon?

Doctor Lee: Yes, we can, but it is difficult to see as complete a vascular network as one can see in the eye vessels. Hairpin loops are primarily arteriolar or muscular in type and compare poorly with vessels in the conjunctiva and in the mesenteric structures of animals, where the anatomy and the responses to drugs are closely comparable. We have completed a study, with regard to the

vasoconstriction and slowed flow that one sees in hypertension in the conjunctiva and in the nail bed, and have found that, if we see phenomena in the eye, another independent observer also notices them in the nail bed. Oddly, in migraine headache, where there may be profound phenomena on one side, these changes also appear in the capillaries of the fingernail bed on the same side as the headache.

Doctor Johnstone: How about the transparent ear chamber in the rabbit? Would that be a good technique?

Doctor Lee: Yes, if you can get the lid off, and if you stimulate with a microelectrode, applying the electrodes to the mouth and the ear vessel.

Doctor Marjorie B. Zucker: The platelets, of course, are important in arresting hemorrhages when vessels are actually severed. Can you see whether they play a part in stopping petechiae formation, the hemorrhages from the dilated sac of normal venules?

Doctor Lee: There is no question but that they play a very prominent role in correcting or attempting to prevent hemorrhagic diathesis. When a peripheral vessel, arteriole or venule, is traumatized, one of the first thing one sees is the collection along the inside of the vascular wall of amorphous material to which platelets immediately adhere. There also appears a prominent constriction, particularly if the vessel is severed. In certain instances, it is possible for a peripheral vessel to bleed prominently and yet have no known defect in the clotting mechanism or the vascular wall *per se*. This results primarily from an inability of the arteriole to constrict down so that you can get a very severe hemorrhagic diathesis. I can at least theorize that, if one were deficient only in some vasoconstrictor principle and yet had a full complement of all elements that control not only intravascular thrombosis but the integrity of the vascular wall *per se*, it would not be unlikely that at least a part of the role of these agents that are protective against hemorrhage, might be that of supplementing vasomotor tone.

STRUCTURAL MAKEUP OF CAPILLARY WALL*

By Benjamin W. Zweifach
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Changes in the functional behavior of the smallest subdivisions of the vascular tree have been related, in the past, only in broad generalities to particular structural elements. This is unfortunate, since the basic function of the circulatory system, the establishment of tissue homeostasis, resides primarily in processes unique to the capillary bed proper. The entire series of structural elements encountered in exchanges between the blood and the tissue cells are collectively referred to as the *hematoparenchymal* barrier. This ubiquitous entity is concerned with a wide spectrum of physiological and pathological processes ranging, on the one hand, from its role as a semi-permeable membrane in the exchange of fluid and solutes between the blood and tissue compartments and, on the other, to its participation in local defense mechanisms, hemostasis, leukocytic invasion, *etc.* An adequate understanding of these fundamental processes will depend, in large part, upon our ability to relate them to particular constituents of the barrier. A singularly rewarding approach to the problem was found to be direct microscopic visualization of the smaller blood vessels in the living animal, using a combination of histochemical, micromanipulative, and biological indices to delineate the locus of the derangement involved.

There has been a tendency to ascribe the functional attributes of the blood-tissue interchange to properties of its most obvious constituent, the endothelial cell. More careful analysis indicates that the endothelial membrane *per se* represents only a skeletal framework onto which are superimposed, on either side, discrete structural entities equally important as determinants of the functional characteristics of this complex barrier.[1] Materials permeating from the bloodstream to the tissue cells encounter five separate structural components: (1) an adsorbed layer of protein lining the inner surface (presumably a plasma constituent and/or blood platelets enmeshed in the pores of the intercellular cement); (2) the endothelial membrane proper, whose surface represents a dual entity, a composite of living cells, and (3) the intercellular cement substance, a small fraction of the total surface; (4) a condensation of fine connective tissue fibrils enmeshed in a dense amorphous ground substance, referred to as the pericapillary sheath; and (5) a layer of connective tissue about 25 to 50 micra in depth intervening between the cell and the vessel proper.

The movement of materials across this barrier is governed primarily by physicochemical forces, with the net exchange across a unit area of capillary surface being determined by intrinsic factors, such as the nature of the pores in the separate structures, the relative thickness of each component, and the lipid solubility of the material involved. The actual surface across which the exchange occurs consists of an extensive cellular area with a small nonliving intercellular zone (less than 1 per cent). There is some disagreement

* This investigation was supported by a research grant from the Life Insurance Medical Research Fund, New York, N. Y.

whether the bulk of the exchange occurs across the entire surface area[2] or is restricted for most molecules to the more pervious intercellular cement.[3] The evidence is clear that the penetration of large molecular aggregates and formed elements probably occurs through the intercellular portion of the wall. In essence, the basic structure involved is a porous network of a complex cement substance, presumably a calcium proteinate. Superimposed on this network either by electrical, chemical, or surface tension forces is a large molecular component which plugs most of the large pores in the cement, considerably reducing the over-all perviousness of the structure. Whether the secondary layer represents a component of the blood-clotting system, as believed by different investigators,[4] or is a combination with blood platelet elements,[5] remains speculative. The increased capillary permeability that develops in conditions involving blood platelet depletion or a defect in the blood-clotting system[6] is suggestive of a direct relationship between these processes and the perviousness of the capillary wall.

The intercellular cement, a structure analogous to the extraneous coats observed in unicellular marine forms, is secreted continuously by the endothelial cell, a major contribution to the integrity of the vascular barrier. In experiments where the capillary was exposed to a weak solution of silver nitrate introduced with a micropipette, the cement material was precipitated, apparently as a silver proteinate. When done carefully, this staining can be achieved without interrupting the blood flow through the capillary vessel. The blackened cement is gradually washed away by the flow of the blood. Since this occurs without affecting the integrity of the vessel wall, it is reasonable to assume that the intercellular material is being replaced by the endothelial cells. In instances where the endothelial cell is damaged either chemically or by mechanical handling, the progressive washing away of the nitrate blackened cement is accompanied by a disruption of the wall and the outward penetration of red blood cells. Stasis then ensues rapidly.

The properties of the cement will vary with changes in electrolyte concentration. For example, the presence of calcium has been found to be important for the maintenance of the normal adhesiveness and tensile strength of this material. In perfusion experiments where the calcium content of the circulation is artificially reduced, the cement appears to go into solution and is washed away. In tissue-culture preparations, the growth of endothelium in the form of sheets or tubes is likewise dependent on the presence of adequate amounts of calcium.[7] For example, when the calcium content is reduced, endothelial sheets begin to fall apart, the cells round up and assume ameboid characteristics or lie rounded in the clot. With the reintroduction of calcium, these cells again flatten out and join with one another to form continuous sheets or tubes. Apparently, a small amount of calcium (about 10 per cent of normal) is sufficient to maintain cellular adhesiveness. In the calcium-deficient experiments, the endothelial cells could readily be detached from one another with microneedles. This is in contrast with experiments using normal concentrations of calcium, where it is almost impossible to separate two contiguous endothelial cells with microneedles without destroying the cells.

The substitution of magnesium or strontium is not adequate to replace calcium with regard to this function. In perfusion experiments, where the concentration of calcium was minimal or absent and the amount of potassium was increased, the intercellular cement of the blood capillaries underwent swelling and became unusually prominent. Under these conditions, the vessel was highly permeable to the extent that edema developed. Extravasation of blood cells was present but not excessive. The use of magnesium ions as a substitute for calcium results in the transformation of the cement into a jelly-like, clear, extracellular material on both the surface of the cell and along the intercellular margins. The presence of excess calcium in perfusion media results in a heavy precipitation along both the intercellular margins of the endothelial cells and the inner surface in contact with the perfusate. It is interesting to note that excessive amounts of calcium will result in the transformation of extraneous coats of various cells from gelatinous materials into a brittle, opaque substance which cannot be stretched by microneedles. Actually, in perfusion experiments with excess calcium (two times normal) the vessels are more easily ruptured by stretching with microneedles than in experiments with normally balanced perfusates.

The intercellular cement material is difficult to visualize under normal conditions. It can be stained, of course, with silver nitrate. In the living vessel, the cement can best be visualized by virtue of its adhesive properties through the intravenous administration of an inert particulate substance, such as carbon or graphite. Under normal conditions, the sticking of carbon is confined to the intercellular cement material (FIGURE 1). Reports indicate that it is possible to stain the intercellular cement with indigo tetrasulfonate methylene blue.[8] Whether this represents a staining of some blood protein which is adherent to the cement or the cement substance *per se* cannot be ascertained with this procedure.

The evidence with respect to the behavior of the cement substance to electrolytes and changes in pH would appear to indicate that the material is a

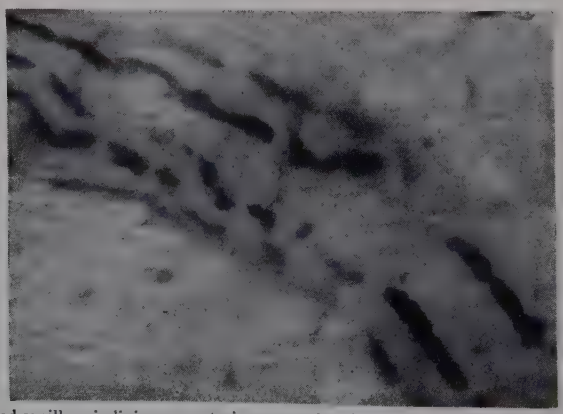

FIGURE 1. Blood capillary in living mesenteric preparation showing intercellular lines following intravenous injection of suspension of fine carbon particles. Carbon adheres only to cement between cells. ×450.

complex protein, possibly a calcium proteinate, differing in chemical and physical properties from that of the connective tissue ground substance.[3] The introduction of a proteolytic enzyme such as crystalline trypsin results in a breakdown of the intercellular cement substance. This is in contrast to the effects of a testicular extract with hyaluronidase activity, where an effect can be demonstrated only on the connective tissue ground substance and on the perivascular sheath.[9] The interendothelial cement appears intact, as evidenced by its reaction to particulate matter and its response to stretching with microneedles. Thus far, in all experiments where the permeability of the capillary as a whole has been increased so as to facilitate the permeation of blood proteins or blood cells, a concomitant change in the physical characteristics of the cement substance has been noted. In general, factors that tend to loosen the cement or to cause it to swell increased the perviousness of the vessel wall, whereas factors which tend to insure the stability of the cement reduce the tendency toward abnormal capillary permeability.

The endothelial cells proper obviously constitute an important structural element from a variety of considerations.[10] First, the tone or elasticity of the capillary wall is, in part, a consequence of the tone of the endothelial cell proper. The tone of nonmuscular cells varies in large part by changes in the water content. Under different conditions, the endothelial cell becomes swollen in appearance and loses its normally elastic properties. This deficiency can be demonstrated by micromanipulative means. When examined under high microscopic magnification, the endothelial cell can be seen to exhibit a constant ameboidlike activity. In conditions where the cell tone is deficient, this activity is lost.

A second phenomenon in which the endothelial cell participates is the continuous replacement of the intercellular cement, as noted previously. Third, the exchange of lipid-soluble materials would appear to be a function of the endothelial cell proper. It is obvious that factors that interfere with the functional state of the endothelium will influence this type of exchange. The requirement of the tissue cells for oxygen and the effective removal of CO_2 are probably referable to this particular phenomenon.

Previous studies have tended to emphasize the extreme thinness of the endothelial cell as an important factor in the exchange of materials between blood and tissues. The fact by itself cannot account for the type of permeability exhibited by the vessel wall. Histochemical studies have indicated that the endothelial cells have an unusual content of glycogen.[11] Recent histochemical investigations with tetrazolium salts have indicated that the endothelial cells are metabolically active (FIGURE 2), the relative order of their activity being comparable to that of vascular smooth muscle.[12] The enzymatic reduction of tetrazolium by endothelium apparently varies with the functional state of the vessel.

Endothelium therefore appears to represent an extremely labile tissue even on a metabolic level. The endothelial component of the blood-tissue barrier is affected by a wide variety of conditions, especially by changes in local tissue metabolism. Hypoxia *per se* does not appear to represent an important con-

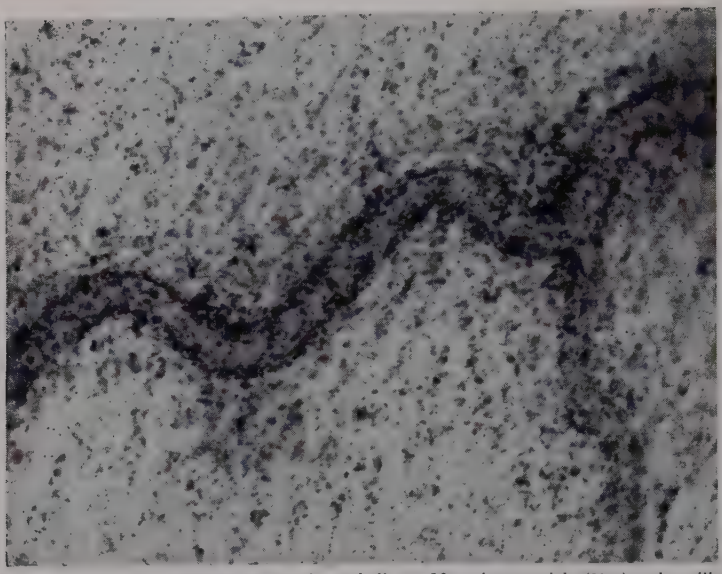

FIGURE 2. Histochemical reflection of vessel metabolism. Muscular arteriole (20 μ) and capillary branch (right-hand side of photo) in mesentery of rat. Vessels colored by reduced neotetrazolium (NT) crystals in smooth muscle and endothelial elements. Living mesentery incubated in NT for three hours at 37.5° C., fixed in neutral formalin. ×100.

sideration, since the oxygen tension of perfusion mixtures must be reduced to almost zero before an effect on the integrity of the endothelium becomes apparent. Other factors, such as the tonicity of the extracellular fluids and pH would appear to be of direct importance. For example, the local introduction of hypertonic solutions (NaCl, glucose, sucrose, albumin) will result in a shrinkage of the endothelial elements and a consequent pulling apart of contiguous surfaces of endothelial cells. This result, of necessity, is accompanied by increased outward loss of fluid and even by extravasation of red cells.

An aspect of capillary structure which has been almost completely neglected is the presence on the inner surface of the endothelial wall of a thin layer closely adherent to the endothelium and the cement.[1] This structure is so thin as to be invisible through the microscope in all but exceptional circumstances. The lining material would appear to represent some constituent of the blood which is deposited onto these surfaces. The exact factor that is involved has not been identified. Because of the intimate relation of the inner-vessel surface to local thrombus formation, together with the adherence of blood platelets to the intercellular cement, the blood proteins concerned with the clotting mechanism have been suspected in this regard. Perfusion experiments, in which the blood colloids were replaced by various synthetic or naturally occurring colloidal agents, indicate that the permeability characteristics of the capillary wall can be altered in this manner. This change occurs independently of the precise osmotic properties of these colloidal agents. The substitution of different colloidal substances as the material plugging the interstices or pores of the capillary membrane will affect its permeability characteristics. Such a role has been

demonstrated not only for a native protein such as albumin, but for substances such as gelatin and gum acacia. In experiments where the capillary system was perfused with colloid-free mixtures, the naturally occurring lining of the vessel could be washed out and the progressive edema studied. The addition of different proteins or colloids to the medium could then be used to study the restoration of normal permeability characteristics. Crystalloidal solutions were completely ineffective.[13] Small amounts of colloid produced an effect on permeability disproportionate to their osmotic pressure values. The addition of blood platelets to the perfusion fluid likewise served to restore partially the permeability of the capillary wall.[14] The platelet factor has been suggested to operate by virtue of a true plugging of the capillary pores. The mechanism, however, is not a simple mechanical effect, since the platelet action is dependent on the presence of other serum proteins and, apparently, upon the physical characteristics of the vessel surface. Thus, this phenomenon could be regulated not so much by the total number of platelets, as by a precise physicochemical relationship between these elements and the vessel wall.

Other investigators have shown that it apparently is possible to displace the normally present lining by the administration of surface active materials.[15] Among the agents found to exert their effect by this particular mechanism are basic proteins, such as clupein. The displacement of the protein lining by these agents results in an altered permeability of the vessel wall. Experiments with heparin also point to an action on the lining proteins leading to an increased capillary permeability.

A prominent feature of the capillary wall is the presence of a sheath of delicate fibrils in the form of a membraneous structure adherent to the outer endothelial surface. This sheath varies in consistency and thickness in different regions as well as under various conditions. Being a condensation of the ground substance of the connective tissue proper, it presents a definite barrier to the passage of substances to and from the capillary lumen (FIGURE 3). It is interesting to note that leukocytes, upon passage through the capillary wall, at first lie in the space between the endothelium and the pericapillary sheath. Red blood cells usually enter the tissue spaces under conditions which weaken this supporting structure. Actually, the most common forms of increased capillary fragility are a consequence of a disturbance in the capillary sheath.

The perivascular sheath undergoes changes in its physicochemical characteristics, in addition to those in the tissue ground substance, and is profoundly altered by enzymes in extracts with hyaluronidase activity. In vitamin C deficiency states in the guinea pig, this structure is likewise found to be deficient. The intravenous injection of snake venoms results in numerous petechial hemorrhages, again believed to be due to alterations in the perivascular sheath.

The final structural component involved in blood-tissue exchange is the connective tissue proper. This structure presumably has no major influence on diffusion processes. The injection of diffusible dyes into the ground substance of the connective tissue results in a progressive, even diffusion similar to that observed when such dyes are introduced into a block of gelatin. Attention should be given to a number of features which could limit the free movement of

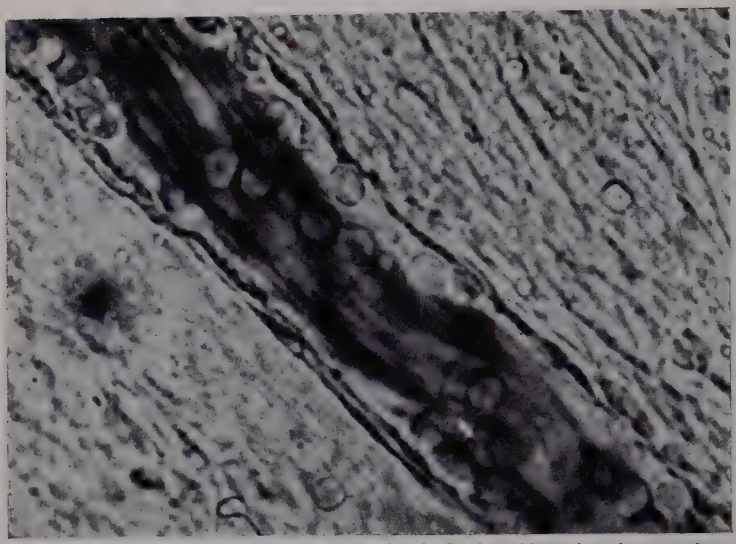

FIGURE 3. Venule shown to illustrate perivascular sheath clearly evident along lower surface of vessel. Photograph of untreated mesentery of rat. ×200.

water and dissolved substances in this medium. It is well known that in hypothyroidism, mucoproteins appear which have an avidity for water and result in a typical tissue edema. It is probable that the avidity of the various fibrous elements and the ground substance for cations may change under different conditions. This change, in turn, can conceivably affect the adsorption of substances such as the hyaluronate compounds to the connective tissue matrix and thereby alter the permeability characteristics of this barrier. Apparently, ascorbic acid deficiencies[16] and X radiation[17] will give rise to abnormalities in the ground substance that lead to increased capillary fragility and edema. The principal influence of a change in the connective tissue proper on capillary permeability would appear to be indirect, resulting from the subsequent disturbance in the metabolic activity of the parenchymal cells proper or of the capillary endothelium.

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Discussion of the Paper

Doctor Szent-Györgyi: Have you ever tried to work to higher magnification, as with the electron microscope?

Doctor Zweifach: We have not, but others have performed studies on muscle, especially since muscle tissue does lend itself to electron microscopy in terms of sectioning. Other tissues are more difficult to prepare, and one can identify the cement and the so-called bridges that hold the cells together. Other cellular details are not too illuminating with respect to the particular problem.

Doctor Bruno Kisch: Working in recent years on electron microscopy, especially of the heart muscle, we could not avoid seeing the capillaries and their structure and, even though that is only a beginning of the investigation, we can say that the capillary itself has a wall that is very complicated in its structure. Sections through a capillary show one wall of the capillary, not the capillary itself. You see here an inner structure, a wall, and an outer structure enclosing a kind of material that, up to now, I have not been able to analyze clearly with the electron microscope, but it always shows in good pictures of the capillaries. You have here, then, the nuclei of the endothelium and the other nuclei and, outside of the wall, very definite sheaths can sometimes be seen running parallel with the capillary; so that a capillary wall is not simply a sheet of tissue, but a very complicated structure. A long time will be needed before we really know this structure and its changes in abnormal conditions of the capillaries. This is a big project that is beginning to show how the electron microscopic structure of the capillary wall changes under certain conditions, especially in vitamin C deficiency.

THE ROLE OF FLAVONOIDS IN COUMARIN ANTICOAGULANT THERAPY*

By Charles E. Brambel
Anticoagulant Clinic, Mercy Hospital, Baltimore, Md.

Evidence is steadily accumulating that carefully controlled coumarin anticoagulant therapy may be advantageous to patients in several major clinical categories. Capillary bleeding is still a sporadic complication and potential hazard associated with the use of these drugs. For example, Prandoni and Wright[1] noted hemorrhage in 8 of 20 patients to whom Dicumarol was given. Purpura, sublingual ecchymosis, conjunctival hemorrhage, gingival hemorrhage, epistaxis, hematuria, bleeding at the site of a wound, and hematemesis were noted. Wright[2] stated that "it is relatively common to find the initial evidences of bleeding in the lower extremities proximal to the ankle in ambulatory patients who are on dicoumarol. One can often see petechial hemorrhages in that area as soon as a few extra red cells appear in the urine or even before." In our experiences, fortunately, the incidence has been and continues to be small. The etiologic factors responsible are not clear, but may be associated with nutritional problems. It has been asked,[3] "Can it be that the normal integrity of the vascular wall is dependent upon a normal process of coagulation and that when this process is accelerated, intravascular clotting occurs and when it is retarded, abnormal bleeding appears?"

Speaking of blood coagulation and of vascular injuries in Dicumarol conditions, Smith[4] conjectured, "It may very well be we are approaching something functionally like thrombocytopenic purpura, with platelets still present but not functioning in the way they should to agglutinate and stop up small bleeding points from the capillaries."

Whatever the causative factor or factors, this erratic change in the vascular wall is distressing to the patient and to the physician as well.

Any supportive measure that could be used to offset the tendency to capillary bleeding would be of great value in the therapy of thrombotic diseases with anticoagulant drugs. Bioflavonoids in conjunction with ascorbic acid offer such a promise.

No satisfactory laboratory test exists which will predict patients disposed to capillary bleeding. The reliability of the various techniques for measuring capillary resistance has been questioned. The tests leave much to be desired, since they are but crude approximations of capillary integrity. We have used the Göthlin[5] infradiastolic pressure test in all of our studies. Experience has shown that many positive tourniquet tests (various modifications) will show no evidence of capillary bleeding, whereas some patients with extensive peripheral hemorrhagic manifestations will show a negative test. Therefore, supportive prophylaxis or immediate therapy with effective agents is the only recourse at present.

Ambulatory patients with a clinical diagnosis of chronic recurrent thrombo-

* Hesperidin-C was furnished through the courtesy of Doctor Gustav J. Martin, Research Director of the National Drug Company, Philadelphia, Pa., and Steven Horoschak of the same company.

phlebitis, cerebral thrombosis, and coronary thrombosis comprise the majority of subjects visiting the Mercy Hospital Anticoagulant Clinic for long-term anticoagulant therapy. The ages range from 25 years to 85 years. The largest number of patients are in the fourth and fifth decades. There is an equal distribution of the sexes.

It is not the province of this presentation to enter upon a discussion of the clinical rationale or value of prolonged anticoagulant therapy. The purpose is to show that such a regimen is possible and the problems which are encountered.

Two thousand patients have been studied in the last 11 years with varying degrees of anticoagulation from 1 month to 9 years. The current census is 600 patients. Out of this group, there was a total of 5 per cent of bleeding complications of varying degrees of severity. One per cent required interruption of anticoagulant therapy with counteractives (vitamin K_1 emulsion intravenously).

Coumarin anticoagulants (Dicumarol, Tromexan, phenylindanedione, Cumopyran, and Marcumar) were administered to maintain a clotting level of two to two and one-half times normal. This level has been found satisfactory from a therapeutic point of view. It is the degree of disruption of the blood-clotting mechanism recommended by the Committee on Anticoagulants of the American Heart Association. The patients were checked at weekly intervals for the blood-clotting value by accepted procedures.

The following types of complications were encountered: (1) discolored purpuric areas on arms and legs appearing spontaneously and without trauma; (2) nose and mouth (gum) bleeding; (3) hematuria; and (4) rectal bleeding. The incidence in categories Nos. 2, 3, and 4 was 0.2 per cent of all the cases. The vast majority of patients evidenced only one episode of purpuric bleeding regardless of severity during the entire period of anticoagulant therapy. About 1 per cent of the bleeding cases showed several recurrences.

The occurrence of capillary weakness with blood extravasation was not related directly to the degree of disruption of the blood clotting mechanism by the coumarin anticoagulants nor to the duration of therapy. It must be stressed, however, that in cases where the anticoagulant mechanism is altered drastically either by hyperreaction to the drug or as a result of accident, capillary breakdown is inevitable. This phenomenon is a manifestation of toxicity to the coumarin drugs. This discussion pertains only to meticulously maintained therapeutic anticoagulation levels.

Vitamin C subnutrition was presumed to lower capillary resistance, but this assumption was not substantiated by investigations[6-9] that showed that capillary resistance tests could not be related to states of vitamin C subnutrition.

Vitamin C is accompanied in nature by another substance which was isolated by Szent-Györgyi[10] and called "citrin." The active principle of this substance was found to be hesperidin, which has been found to have the capacity to correct capillary fragility and permeability.

Two types of study were undertaken:

(1) *Prophylactic.* The routine administration of bioflavonoids with ascorbic acid to an unselected group of patients, together with an increase in the depression of the blood coagulation mechanism (three to three and one-half times

normal). It was felt that a sample of 200 patients for two months would be sufficient to give an indication of the effectiveness of this combination. These patients received one tablet containing 50 mgm. each of hesperidin and ascorbic acid three times daily. There were no hemorrhagic complications of any kind in this group of patients at the end of this test period. This finding suggests that Hesperidin-C may decrease the incidence of hemorrhagic complications sometimes encountered during anticoagulant therapy, and the patients' tolerance to greater depression of the blood coagulation mechanism was increased.

Since extremely drastic depression of the clotting mechanism is not desirable as a therapeutic measure, and as the incidence of hemorrhagic complications is low, prophylactic therapy with bioflavonoids and ascorbic acid is not too urgent.

(2) *Therapeutic.* Administration of Hesperidin-C upon the immediate onset of capillary leakage, regardless of severity or type. The dosage of Hesperidin-C was two tablets, four times daily. The ecchymotic areas cleared very rapidly (usually in two to three days). The anticoagulants were not discontinued except in severe cases (gross hematuria or excessive nose bleeding). Before the availability of Hesperidin-C as a supportive medication, the purpuric areas persisted for much longer periods and required discontinuance of coumarin drugs.

From the foregoing studies, it becomes apparent that, if the hemorrhagic hazards of long-term anticoagulant therapy are to be minimized, supportive measures are desirable. Such a measure is found in the availability of a combination of bioflavonoids and ascorbic acid. The preparation that has been found to give reasonably reliable results is Hesperidin-C. Hesperidin alone is less effective, as is ascorbic acid given by itself.

We agree with other investigators[11-13] that a combination of the two compounds is essential for correcting abnormal capillary function. It is tempting to suggest that, since the bioflavonoids are good antioxidants, they serve as sparing agents for ascorbic acid.

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Discussion of the Paper

Doctor Lampen (*Squibb Institute for Medical Research, New Brunswick, N. J.*): How large a group of patients is involved in the actual therapeutic test described here as against the group, apparently about 2000, that actually received the anticoagulant?

Doctor Charles Brambel: The number of patients is, of necessity, small because they received medication only as indications occurred. Of the present group of 600, I do not have the current statistics for the past year at my finger tips. I should say that patients exhibiting very minor ecchymosis would be less than one tenth of 1 per cent. The figures I gave you were based on the over-all 2000.

Doctor Lampen: Was the group treated with Hesperidin-C, therapeutically, less than one tenth of 1 per cent?

Doctor Brambel: Those that we should see would be those with the purpura, *i. e.*, less than 1 per cent. If we see one patient in a month we do very well.

Doctor Sevag: Have you observed positive results in all the patients you have treated?

Doctor Brambel: All the patients were positive. They cleared up very rapidly.

Doctor Sevag: How many patients?

Doctor Brambel: I should say there were about 20 therapeutically treated patients in all, because patients in this category are those that have scleral hemorrhages, or petechia of one kind or another, or hematuria. We do not see large numbers of patients, but they are treated immediately. We have a census of 600 patients, of whom we see about one a month.

Doctor Carson: Was the Hesperidin-C combination ever given with vitamin K, and if so, what results did you get under those conditions?

Doctor Brambel: First I should like a definition of what you mean by vitamin K. If you mean methyl naphthoquinone, that is the preparation that we have tried. I could see no very great function of methyl naphthoquinone in the combination as we used it, therefore I cannot answer the question directly. I should say that one could not see the effect unless methyl-phytyl naphthoquinone were used. In a patient with severe hemorrhage, the practice is to give methyl-phytyl naphthoquinone immediately and massive doses of Hesperidin-C. I am not prepared to say what it does, where the clotting mechanism of vitamin K_1 is, or whether it is potentiated by the flavonoid.

Doctor Swazer: Would you expand a little on the number of patients that

did not tolerate ascorbic acid and about the magnitude of the dose in those that did tolerate it.

Doctor Brambel: In the last six months we have encountered five patients that did not tolerate oral doses of 100 mg. of ascorbic acid daily without severe headaches and other effects that go with it. We have not tried intravenous doses. These patients will tolerate up to 600 or 700 mg. of ascorbic acid when given a flavonoid such as hesperidin.

Doctor S. Shapiro (*New York University Medical School, New York, N. Y.*): Have you made any study to determine whether or not these factors influence the hypoprothrombinemia induced by a fixed dose of any of these various agents, for example, Dicumarol?

Doctor Brambel: We have maintained a fixed anticoagulant level by giving a variable dose.

Doctor Shapiro: How often do you see a patient?

Doctor Brambel: Every week.

Doctor Shapiro: You can shift the dose?

Doctor Brambel: The dosages vary from week to week, and the dosage pattern within the week also varies.

Doctor Shapiro: Let us assume you gave a patient half a gram of Dicumarol, and saw him a week later. What would be the prothrombin time? You give daily doses, is that the idea?

Doctor Brambel: We have a very labile pattern. The dosages are given no more often than daily, and then on alternate days, when a larger dose than the individual daily dose is given.

Doctor Shapiro: Let us assume you saw the patient in a week, and the prothrombin time was just moderately elevated, then would you increase the dosage schedule?

Doctor Brambel: Yes.

Doctor Shapiro: If you see a patient only once a week, how can you determine what the prothrombin time is during the week compared with the period when he is not receiving these factors? To make your experiment valid, would you not have to make at least daily estimations for an appreciable period of time to determine whether or not these factors influence the induced hypoprothrombinemia?

Doctor Brambel: I agree with you. We have been working in this field for practically 14 years. We spent five or six years in studying patients daily, every other day, and weekly. On a basis of the statistical response pattern now based upon our accumulated data and random sample checking of these patients, our predications come out very close. In other words, that is precisely why we are interested in some of the newer anticoagulants, where the sustained anticoagulant effect loses some of its variability. We have checked patients in the middle of the week beforehand and it has been worked out statistically. It is our routine pattern to make such checks on patients in the middle of the week to be sure.

With regard to the appearance of hemorrhagic complications, we guard against missing them during the middle of the week because the patients are all

very well educated. At the first sign of any hemorrhagic complication, they promptly call us on the telephone and, if there is any question at all regarding the degree of severity, the patient is invited to come in to be checked. So we keep very strict and close supervision of all of our patients.

Doctor Harold Neumark: Have you checked any patients with hemorrhagic complications following heparin therapy rather than Dicumarol therapy? That is, have you tried vitamin C, hesperidin, on coumarin-treated patients? Has such treatment worked at all on heparin-treated patients?

Doctor Brambel: Our experience with heparin is extremely limited. We would give it only to hospitalized patients. The occurrence of hemorrhagic complications following the use of heparin are not seen in short-term treatments. Usually, when such complications occur, there are other very severe derangements in the clotting mechanism that necessitate counteraction of the heparin effect.

Part II. Clinical Studies

RHEUMATIC FEVER: OBSERVATIONS ON THE HISTOGENESIS, PATHOGENESIS, AND USE OF ASCORBIC ACID AND BIOFLAVONOIDS*

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Our initial interest in rheumatic fever dates from 1934, when we reported the development of lesions resembling those of rheumatic fever in the heart and articular tissues of guinea pigs subjected to scurvy and hemolytic streptococcal infections.[41, 39] Subsequently, Stimson, Hedley, and Rose[46] reported the finding of a proliferative endocarditis in guinea pigs subjected to scurvy and injected with a streptococcus toxin. Our experimental observation was confirmed by Schultz,[43] although he did not consider that the lesions produced were characteristic of rheumatic fever. Others[26, 48] recorded the occurrence of degenerative and proliferative reactions in the heart valves of scorbutic guinea pigs in which a factor of infection was not introduced, and they observed no clear difference in the animals subjected to added infection. We had encountered mild reactions of this type in scurvy, but the distinctive lesion of rheumatic character occurred only in those animals subjected to combined scurvy and infection. The investigators cited, however, used hemolytic streptococci derived from human sources, which are not natural pathogens in the guinea pig, while we employed a group C hemolytic streptococcus, a natural pathogen in this animal. In view of subsequent studies, it is also of interest that the organism that we used possessed a prominent mucoid capsule.

We have previously reviewed studies of our own and others[35] directed at the determination of the nutritional status of individuals suffering from rheumatic fever. These data indicated the very common occurrence of degrees of vitamin-C deficiency at the time of onset of the disease. Administration of vitamin C in usual doses does not exert a direct curative action or serve to prevent recurrence of the disease.[22] This property does not imply, however, that it is without therapeutic value. Jones[19] noted the recent marked reduction in the incidence of distressing hemorrhagic manifestations of rheumatic fever, *i.e.*, epistaxis and purpura. He noted that "Ten years ago in a ward of 7 or 8 children with active rheumatic fever, 3 or 4 nasal packings daily were frequently necessary. This picture is now completely altered. Nose bleeds are so reasonably mild and less frequent that packing of the nose is unusual." It seems quite likely that this improvement is due to the more liberal and intelligent use of vitamin C-rich foods or ascorbic acid in the management of cases. Important relevant clinical studies by Glazebrook *et al.*[11, 42] have escaped the attention which they appear to deserve. These observations afford direct clinical evidence that vitamin-C deficiency may be a major disposing influence in the pathogenesis of rheumatic fever.

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In 1939, Roff and Glazebrook[42] described cases of gingivo-stomatitis among boys in a training establishment of the Royal Navy. "The gums were congested and spongy, the surfaces having a gelatinous feel. Bleeding did not occur on simple palpation, but if one pierced with a probe the haemorrhage was more copious than usual. The congestion was uniform, from the gums into the sulci on to the buccal mucous membrane, extending backwards and involving the tonsils and pharyngeal wall as far as the eye could see." In all cases vitamin-C deficiency was found, with an average ascorbic acid deficit of approximately four grams. The condition responded to administration of ascorbic acid. These authors also recorded prominent symptoms of lassitude with rheumatic pains in and around the larger joints, and noted that exactly similar symptoms occur in cases in which there is evidence of infection, and that such cases may later develop rheumatic fever, with true arthritis, or that carditis may develop silently without further manifestations of rheumatism. They state: "It is often impossible to differentiate from the description of the symptoms of the patient a case which will clear up on saturation with vitamin C, from one which will tend to progress to rheumatism and carditis."

In a subsequent report, Glazebrook and Thomson[11] recorded observations of unusual interest. The study was conducted at a training school for young men between 15 and 20 years of age. The circumstances presented a unique opportunity for study of hemolytic streptococcic infection in a population that might be considered to be in a state of subclinical scurvy. It was estimated that the average daily intake of vitamin C per student was between 10 and 15 mg. Recurrent waves of hemolytic streptococcic tonsilitis afforded the factor of infection. Of the approximately 1,500 students observed in the study, 335 were given liberal daily supplements of ascorbic acid, which was added to milk and cocoa. In sample studies, it is noteworthy that these youths required a total supplement of approximately four gm. of ascorbic acid to achieve saturation. This is the approximate tissue depletion in cases of clinical scurvy. No significant difference in *incidence* of common colds and tonsilitis was demonstrated. The duration of illness with the common cold was not different in the two groups, averaging 6.3 days in the vitamin C-treated classes and 6.4 days in the controls. The *duration* of illness due to tonsilitis, however, was significantly different in the two groups. In the vitamin C-treated classes, the average stay in the hospital was 10.05 days and, in the control groups, 16.7 days. *The most striking influence of vitamin C was in the reduction of the incidence of two complications of the streptococcic infection. There were 17 cases of "pneumonia" and 16 cases of acute rheumatism among the 1,100 controls and no case of either disease among 335 youths having vitamin C.* Analyses showed that a difference as great or greater than this would be expected once in 50 times in a homogeneous population. The authors felt that there was some relationship between the cases of pneumonia and those of rheumatic fever. They noted the occurrence in the institution of a low-grade basal lung consolidation or pneumonitis that appeared to be related to both rheumatism and vitamin-C deficiency. "It was characterised on the one hand by its tendency to progress into rheumatism, and on the other hand by its disappearance when treated with ascorbic acid. This pneumonitis, apart from a vague picture of ill-health,

gave little clinical evidence of its presence, but it probably predisposed toward the development of acute pneumonia." This remarkable clinical experiment can probably never be repeated. It would seem clearly to indicate that, in the presence of streptococcic infection, vitamin-C deficiency disposes the individual to the development of rheumatic fever.

Hedley[17] has recorded an apparent diminution of the incidence of rheumatic heart disease among persons 5 to 24 years of age during the period 1930 to 1936, compared with 1922 to 1929. It is possible that increased knowledge of the importance of nutrition to health and the emphasis upon the possible role of nutritional deficiency in rheumatic fever may be responsible for this decline.

In 1944 and subsequently,[36] we reported that the administration of vitamin C, together with a mixture of crude hesperidin and hesperidin methyl chalcone,* appeared to exert a favorable effect on the course of rheumatic fever. The study included 39 cases, 24 children and 15 adults. Twenty-six of the 39 cases had shown clinical evidence of persistent activity of the disease process for periods longer than six weeks in spite of the application of the usual methods of management. The average duration of the illness in this group was 10 weeks. The average sedimentation rate of the 39 cases, at the time treatment was instituted, was 33 mm. per hour in terms of the Wintrobe scale. One month from the institution of treatment, the average sedimentation rate was 17.5 mm. per hour. Thirty-four of the 39 cases showed significant slowing of the sedimentation rate at the end of 6 weeks (4 weeks in 12 of the cases in which observation was terminated at the time). In two cases, no significant changes in sedimentation rate were observed. In three, the rate of sedimentation was more rapid. Two of the latter had suffered obvious intercurrent infection. Between 4 and 6 weeks after therapy, 22 of the 39 cases showed either no evidence of activity or minimal activity. The findings in the 26 persistent cases are of particular interest. In this group, the average initial sedimentation rate was 32 mm. per hour and, at the end of 1 month, was 15.5 mm. per hour (Wintrobe scale). It is noteworthy that this occurred, in such a short period of time, in a group of cases that, under observation, had shown persistent activity over considerable periods of time. At the end of 1 month, 22 of the 26 cases in this group had exhibited significant slowing of the sedimentation rate. In three, the rate was more rapid and, in one, it was unchanged. Sixteen cases showed either minimal or no activity at the end of one month. These observations indicate that the slowing of the sedimentation rate was related to the treatment. Emphasis has been placed upon the sedimentation rate in this study because it is the best objective index of disease activity in cases in which the diagnosis of rheumatic fever has been established. In many instances, an accelerated sedimentation rate is the only indication of continued active disease. In so far as other manifestations of activity of the disease existed, improvement occurred which paralleled the slowing sedimentation rate.

Confirmatory evidence of a beneficial effect of bioflavonoids on the course of rheumatic fever was secured in a subsequent study of 31 young adults with the disease.† The 31 cases were divided without selection into "control" and

* Generously supplied by the California Fruit Growers Exchange.

† This study was conducted with the cooperation of the United States Army Air Force at Biloxi, Miss., under the direction of Lt. Col. A. W. Wallace.

TABLE 1

INFLUENCE OF BIOFLAVONOID ON SEDIMENTATION RATE IN RHEUMATIC FEVER

	Controls (17 cases)	Hesperidin-treated (14 cases)
Average sedimentation rate at onset of the observation period	25 mm. (Westergren)	30 mm. (Westergren)
Average sedimentation rate after 1 month	25	14
Average sedimentation rate at 6 weeks (or at the termination of observation period)	19.7	13.7
Average slowing of sedimentation rate during observation period	5.3	16.3
Sedimentation rate at end of observation period (more rapid)	4 cases	none
No change	5 cases	2 cases
Slowing of sedimentation rate 10 mm. or more at 1 month	5 cases	11 cases

"treated" groups, and observations were made during the same period of six weeks. In a few of the cases, the observation period was only four weeks. Fourteen of the patients were given supplements of hesperidin and hesperidin methyl chalcone, and a group of 17 patients served as controls. The study was limited to observations on the influence of the flavonoids upon the sedimentation rate. No follow-up studies were made. It is recognized generally, however, that the sedimentation rate is a sensitive index of rheumatic activity. The data shown in TABLE 1 indicate that the rheumatic process was favorably influenced.

Kugelmass[21] has reported that administration of vitamin P active substances (bioflavonoids) increased a lowered capillary resistance and diminished the frequency and severity of epistaxis in rheumatic children.

Alterations of Connective Tissue Ground Substances in the Early Lesions of Rheumatic Fever

Delineation of the intimate early morphologic features of a reaction to injury may contribute substantially to an understanding of the nature of a pathologic process. This study is concerned with the earliest detectable tissue reactions in rheumatic fever. It constitutes a major segment of this report.

The concept is generally held that the initial injury in rheumatic fever involves "fibrinoid degeneration" of collagen. Talalajew[47] noted, however, that a "mucinous edema," and Klinge[20] that a "separation of collagen fibres" preceded the fibrinoid alteration. Altshuler and Angevine[4] described an increase of metachromatic staining material at the site of injury before development of the fibrinoid change. This increase they considered to be the result of a precipitation of alkaline protein with the acid mucopolysaccharide of the connective tissue ground substance. Bunting[5] also recorded conspicuous amounts of metachromatic material at the site of the myocardial Aschoff body. It is not the purpose of this report to dwell upon the controversial subject of "fibrinoid" degeneration. In our experience, it is not the initial or even a constant lesion of rheumatic fever.

Until recently, demonstration of the "ground substance" of connective tissue (the mucinous component—an acid mucopolysaccharide) has been dependent upon its metachromatic staining with toluidine blue or thionine. While this reaction has been of great value in the study of ground substance, it is subject to certain vagaries dependent upon the stain, fixation, and other technical variables, and it does not clearly demonstrate the more finely distributed "ground substance."

In addition to toluidine blue, three histochemical staining reactions have been employed in this study. One is based upon the Hale[15] technique, which is dependent upon the affinity of acid mucopolysaccharides for colloidal iron and their subsequent delineation by the Prussian blue reaction with potassium ferrocyanide. In the modification developed by us,[38] which includes appropriate counterstaining with cochineal and fuchsin, the ground substance, basement membranes, collagen fibers and reticulin fibrils, and fibrin can be differentiated. A combination of the colloidal iron staining and the periodic acid-Schiff reaction has also proved useful. A modification of Gomori's aldehyde-fuchsin staining technique,[13] which we believe demonstrates *sulfated* acid mucopolysaccharides and related sulfated substances, has also been used.[1] Pretreatment of sections with testicular hyaluronidase serves to remove much of the acid mucopolysaccharide and has clarified some of the fine histologic interrelationships. It should be recalled that testicular hyaluronidase will not only depolymerize hyaluronic acid but other related mucopolysaccharides. Acid mucopolysaccharides are found intimately related to perivascular connective tissue cells and collagen fibrils, as well as to the delicate reticulum cells and fibrils supporting the heart muscle cells. The heart valves contain considerable amounts of acid mucopolysaccharides, a portion of which appears to be sulfated, as judged by the reaction to aldehyde-fuchsin. Similarly, the acid mucopolysaccharides cementing the cells in the walls of arteries are chiefly sulfated. Our histochemical observations are in agreement with the biochemical findings of Meyer.[28]

The *Aschoff* reaction is a distinctive one and, in its typical form, is probably as "specific" as any histopathologic reaction. It is not necessary to dwell upon the evolution of the "rheumatic" lesion as seen with usual histologic techniques. This has been done—and beautifully illustrated—by numerous pathologists.

In this report, it is our purpose to describe and illustrate the earliest phases of the "rheumatic reaction" as revealed by the techniques applied and to consider the implications of this reaction to the pathogenesis of rheumatic fever.

In the heart valves, the early reaction involves a swelling of the "ground substance" which is normally present in this structure. The swelling of the ground substance is greatest at the "line of closure" of the heart valves. The endothelial surface layer is interrupted, and fibrin (and probably also other protein elements derived from the blood) is precipitated in the swollen ground substance. Coincidentally, there is proliferation of the endothelial and mesenchymal connective tissue cells of the heart valve (FIGURES 1 to 3). These alterations make up the structure of the fine valvular verrucae so characteristic of early rheumatic endocarditis. FIGURES 4 and 5 illustrate the interstitial re-

action in a heart valve prior to full development or preceding the verrucal lesion. In the heart muscle, the reaction is dominantly in the delicate connective tissue which adjoins the small twigs of the coronary arteries (FIGURES 7 to 12). In the earliest phase, the cells are spread apart, accompanied by and probably due to a swelling of the mucoid ground substance that is closely related to the cells and to their very delicate fibrillar cytoplasmic extensions (FIGURE 8). Removal of most of the mucoid substance by pretreatment of sections with hyaluronidase "uncovers" the delicate collagen or reticulin fibres that were ensheathed with the acid mucopolysaccharide (FIGURES 8 and 9). From the histologic evidence we have seen, it seems logical to assume that the perivascular and interstitial connective tissue or reticular cells normally elaborate an acid mucopolysaccharide which surrounds them and the delicate collagenous (or reticulin) fibrils. Reticulin fibrils (or aggregated fibrils—collagen) probably represent a differentiated component derived from the ground substance. The intimate site of the myocardial rheumatic injury, then, appears to be in the mucopolysaccharide component of the perivascular and interstitial connective tissue, and the reaction to this injury is characterized by a swelling of the mucoid substance. Coincident with this injury, characteristic changes occur in the nuclear structure of the adjoining related cells. They become enlarged, and an abnormal chromatin arrangement produces the characteristic "owl-eyed" nuclear structure of the "Anitschkow" cell. We have observed similar nuclear alteration of connective tissue cells in scurvy. Multiplication and fusion of such cells finally results in the formation of a distinctive Aschoff body (FIGURES 10 to 12). So-called "fibrinoid degeneration" is encountered in some of the lesions of rheumatic fever. It should be noted that fibrinoid degeneration is not consistently present and is not the initial manifestation of the rheumatic injury. In the heart valves, "fibrinoid" is most commonly seen in the more superficial portions of the endocardial verrucae (FIGURES 2 and 3). It is present in certain of the myocardial Aschoff bodies (FIGURES 8 and 9) and is particularly prominent in rheumatic pericarditis in the "active" phase (FIGURE 6). The term "fibrinoid" indicates the fibrinlike staining property of the substance. In our studies, "fibrinoid," when present, has been found closely related to small dilated blood vessels or endocardial surfaces exposed to blood. The ribbonlike, highly eosinophilic masses of "fibrinoid" material seen in some lesions of rheumatic fever are believed by the writer to represent fibrin, possibly associated with other protein elements derived of blood, which has been precipitated in swollen acid mucopolysaccharide. We have recently shown that the native mucopolysaccharide of the vitreous humor of the eye accelerates blood clotting.[3] It may be that the swollen acid mucopolysaccharide stimulates precipitation of fibrin and renders it resistant to fibrinolysins. It would not be surprising if this substance lost some of the typical staining reactions of fresh fibrin after a period of time.

It has been reported that the Aschoff bodies of rheumatic myocarditis develop from injured myofibers.[30] While evidence of injury to myofibres is seen occasionally in rheumatic myocarditis, this view appears to be quite untenable to the writer. In the first place, the Aschoff response occurs in sites such as

heart valves and subcutaneous tissues where myofibers are not present and, further, the distinctive site of the early myocardial lesions is in the perivascular connective tissue, clearly separate from heart muscle fibers.

Related Experimental Observations

The character of the early lesions of rheumatic fever just described indicate that the intimate site of injury in rheumatic fever is in the connective tissue ground substance, particularly in sites rich in hyaluronic acid and possibly involving interactions of hyaluronic acid and hyaluronidase. Before considering the implications of this finding and possible relationships to ascorbic acid and bioflavonoids (see discussion), it is pertinent to record in brief a few relevant but unpublished experimental observations which we have made.

Inhibition of spreading reaction by hesperidin methyl chalcone. In recent studies, we[40] have found that the oral administration of hesperidin methyl chalcone to guinea pigs will diminish the spreading reaction of intracutaneously injected hyaluronidase.

Lesions induced by injection of hyaluronidase. In another unpublished study,[2] it was found that repeated intravenous injections of large amounts of testicular hyaluronidase in rabbits produced myocardial lesions which were characterized by foci of mucinous edema and cellular proliferation. In one group, three rabbits were given six injections of 50 mg. of hyaluronidase* at three- or four-day intervals and examined eight days after the last injection. These animals showed multiple foci of mucinous edema of the interstitial connective tissues of the heart, associated with proliferation of the connective tissue cells. The animals also showed mucoid swelling and mild cellular proliferation of synovial tissue. The single control animal in this series did not show such lesions.

In a second, similar experiment, 6 animals received 6 injections of 20 mg. hyaluronidase† over a period of 23 days (4 animals) and 35 days (2 animals). Five were examined at intervals of 5 to 10 days after the last injection. Examination of one animal was deferred to 18 days after the last injection. Similar, though less marked, pathologic alterations were found in this series of animals. Four animals served as controls. In three, there were no comparable lesions.

* Armour testicular hyaluronidase 200 TRU/mg.
† Wyeth 1000 TRU/mg.

FIGURE 1. The mitral valve and adjoining heart muscle from a case of active rheumatic carditis. The mucopolysaccharide ground substance stains blue. It will be seen that the mitral valve has a high content of such material. The bluish foci in the mural endocardium and in the heart muscle are sites of the Aschoff reaction, which are characterized by swelling of the ground substance. A rheumatic verrucous lesion is present on the mitral valve. Stain: colloidal iron. ×4.

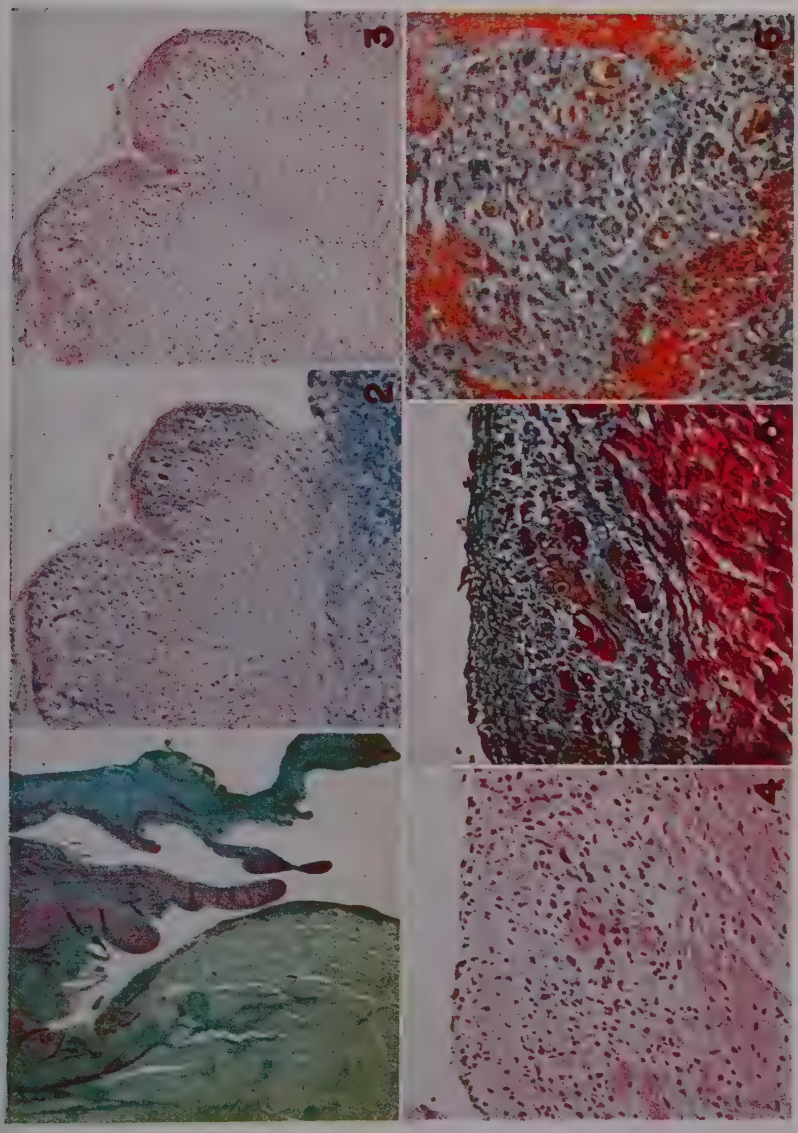
FIGURE 2. Detail of an early verrucal lesion on the mitral valve. The mucoid ground substance of the valve stains blue. The lining endothelium is interrupted. A thin layer of the red-staining fibrin at the surface merges with the swollen ground substance of the valve. Enlarged and irregular cells are proliferating in this mucinous matrix. Stain: colloidal iron. ×160.

FIGURE 3. A section directly adjoining that shown in FIGURE 2. This section has been pretreated with hyaluronidase prior to staining. It will be seen that the mucoid component has been largely removed by this procedure. Stain: colloidal iron. ×160.

FIGURE 4. An early rheumatic reaction in the pulmonary valve from a case of acute rheumatic fever. The surface endothelium is intact, but there is a proliferation of large, irregular cells in the underlying tissue. Stain: hematoxylin and eosin. ×160.

FIGURE 5. A section directly adjoining that shown in FIGURE 4. It will be seen that the cellular proliferation is occurring in the swollen ground substance of the valve which stains blue. Bundles of collagen fibres are stained red. Stain: colloidal iron. ×160.

FIGURE 6. Rheumatic pericarditis. The reactive vascular connective tissue contains much blue-staining mucinous ground substance and multiple orange-red staining masses of hyalin fibrin. Stain: colloidal iron. ×160.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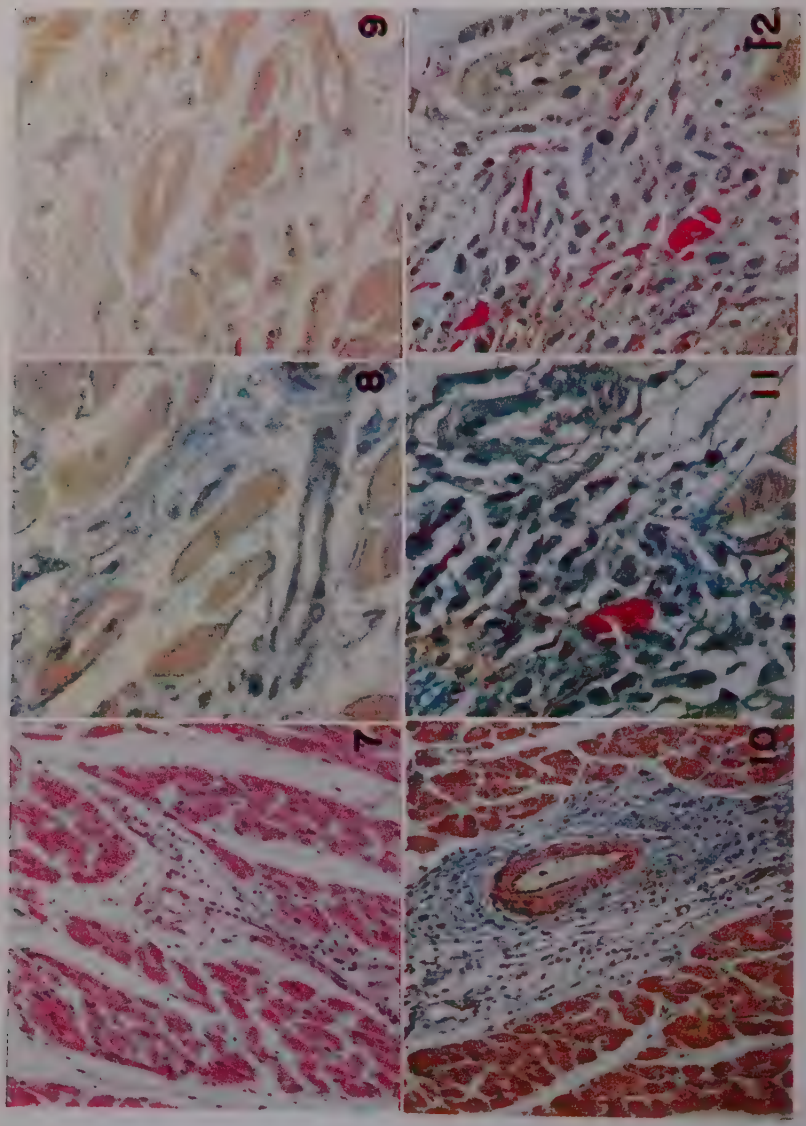
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One control animal which showed a mild myocardial reaction had had a spontaneous ocular and respiratory infection during the experimental period. We do not consider these lesions to be the counterpart of rheumatic lesions, although they do show the common components of ground substance swelling and cellular proliferation.

The influence of hesperidin methyl chalcone on the DOCA-salt injury. Since the original observations of Selye,[44] it has been shown repeatedly that experimental administration of desoxycorticosterone and salt will cause hypertension, cardiac hypertrophy, glomerular injury, and vascular lesions resembling those of periarteritis. Detailed histologic studies have not been made, however, and the pathogenesis of the process has not been clarified. With this in mind, we have conducted experiments utilizing this procedure that will be reported in detail at a later time.[2] However, a brief review of some of our findings seems pertinent to this report.

Rats were unilaterally nephrectomized. Four animals served as "nephrectomy controls." Fifteen-milligram pellets of DOCA were implanted beneath the skin in 20 other animals at the onset of the experiment and, again, two weeks later. The animals were given 1 per cent sodium chloride to drink. Ten animals received 20 mg. of hesperidin methyl chalcone 2 times daily by subcutaneous injection, and 10 animals received the DOCA and salt without other treatment. The experiment was terminated at 35 days. Data regarding blood pressure, body weight, kidney weights, and heart weight are shown in TABLE 2.

It will be seen that the body weight, heart weight, and kidney weights of the animals treated with the flavonoid closely paralleled those of the controls, while the untreated animals showed a lower average body weight and increased kidney and heart weights. The mean blood pressure of the untreated animals was significantly elevated, while that of the treated animals showed little if any rise.

In the unilaterally nephrectomized animals receiving DOCA and salt, the anticipated lesions were found. In the heart, there was a diffuse interstitial myocarditis characterized by swelling of the acid mucopolysaccharides of the connective tissue ground substance and associated cellular proliferation. Arteritis developed in the coronary arteries of 6 of the 10 animals. Eight of the 10 animals exhibited typical lesions of periarteritis in pancreatic arteries, and

FIGURE 7. A very early rheumatic reaction in the heart muscle. The tissue adjoining the small twigs of the coronary vessels is edematous, and there is an early proliferation of the perivascular cells. Stain: hematoxylin and eosin. ×160.

FIGURE 8. A very early rheumatic reaction in the perivascular connective tissue of the heart muscle. The connective tissue ground substance is edematous or swollen, and there is an early proliferation of the associated cells. Stain: colloidal iron. ×320.

FIGURE 9. This section is one adjoining that shown in FIGURE 8. It has been treated with hyaluronidase prior to staining which has removed the mucinous ground substance. This has revealed some very delicate collagen fibrils and has enhanced the nuclear staining of the reacting cells revealing the characteristic "owl-eyed" nuclear structure. Stain: colloidal iron. ×320.

FIGURE 10. This section shows a relatively early Aschoff reaction in the connective tissue surrounding a small branch of a coronary artery. The swollen connective tissue ground substance (blue) is the site of proliferating cells. Stain: colloidal iron. ×160.

FIGURE 11. This section shows a somewhat more advanced Aschoff reaction with prominent swelling of ground substance (blue) and numerous reacting cells. A focus of the fibrinoid changes (orange) may be seen in the upper left portion of the photograph. The red-staining tissue is collagen. Stain: colloidal iron. ×300.

FIGURE 12. This section is one adjoining FIGURE 11. The mucinous ground substance has been removed by pretreatment with hyaluronidase revealing the previously obscured collagen bundles (red). The characteristic nuclear structure of the reacting cells is well shown. The fibrinoid alteration may be seen at the left side of the photograph. Stain: colloidal iron. ×300.

TABLE 2

Group	No. of animals	Initial weight (average)	Final weight (average)	Kidney weight (average)	Heart weight (average)	Final blood pressure (average)
Unilateral nephrectomy	4	252	267	1.150	0.875	115
Unilateral nephrectomy + DOCA and salt	10	265	240	1.450	1.150	155
Unilateral nephrectomy + DOCA and salt receiving HMC	10	255	265	1.200	0.925	118

TABLE 3

Group	No. of animals	Heart Interstitial myocarditis	Heart Periarteritis	Pancreas Periarteritis	Kidney Glomerular lesions
Unilateral nephrectomy	4	0	0	0	0
Unilateral nephrectomy + DOCA and salt	10	8	6	8	4
Unilateral nephrectomy + DOCA and salt receiving HMC	10	2 (mild)	0	0	1

all showed interstitial pancreatitis. The early arteritic lesions were typified by swelling of the mucopolysaccharides in the adventitia and media of small arteries. Later lesions exhibited "fibrinoid" necrosis of the vessel walls and thrombosis. Four animals showed well-defined glomerular injury. The animals receiving hesperidin methyl chalcone were almost fully protected from the lesions of DOCA and salt. Two of 10 animals showed only a mild interstitial myocarditis, and one showed relatively minor glomerular injury. A summary of the microscopic alterations is presented in TABLE 3.

It appears that a primary feature of the DOCA and salt injury is one which involves a swelling of the ground substance (acid mucopolysaccharide) and, in this sense, it bears some analogy to the rheumatic injury.

Discussion

How may we integrate and interpret the observations recorded? The early lesions of rheumatic fever appear clearly to involve the ground substance of connective tissue, particularly that of the heart valves, heart muscle, and joints, all of which contain considerable amounts of hyaluronic acid. The injury is one which causes a swelling of such ground substance and is associated with a distinctive type of cellular reaction at the site of injury constituting the Aschoff reaction. Evidence that the hemolytic streptococcus is in some way related to the etiology and pathogenesis of rheumatic fever seems quite beyond question. This evidence has accumulated over the years and, in recent years, it has been shown that, if infections with this organism can be prevented or treated early enough, the disease may be prevented. It is noteworthy that the beta hemolytic streptococcus is unique in possessing a capsule composed of hyaluronic

acid. Can it be that the body defenses elaborate an agent such as hyaluronidase which may act not only on the bacterial capsule but on the chemically related ground substance of connective tissue—a possibility suggested by Dorfmann?[9] It is possible, too, that an antigenic substance derived from the capsulated hemolytic streptococcus might become incorporated in the ground substance of chemically related connective tissues, and that an interaction with an antibody may release a depolymerizing agent (such as hyaluronidase) causing swelling of the ground substance.[37] Jones[18] has reported that the repeated subcutaneous administration of several mucopolysaccharides in guinea pigs has caused cardiac lesions with some resemblance to those of rheumatic fever. That hyaluronidase may be acting in the pathogenesis of rheumatic fever is suggested by a number of investigations. The increase of an antihyaluronidase (antibody type) in the blood of cases of active rheumatic fever is well attested.[34] A nonantibody antihyaluronidase may be elevated in other disease states as well as in rheumatic fever.[14] Dorfman *et al.*[10] found the nonspecific hyaluronidase inhibitors elevated in a group of young adults in the acute phase of rheumatic fever. Hartmann and Matijevic[16] have recently presented evidence that the blood from patients with acute rheumatic fever possesses *hyaluronidase* activity. The influence of the hemolytic streptococcus in the pathogenesis should not be minimized in any way. It is clear, however, that rheumatic fever is "selective." While respiratory infections with effective strains of hemolytic streptococci are common, in only a small minority of cases are such infections followed by the rheumatic syndrome. The difference in frequency of rheumatic fever at the extremes of the social economic scale has not received the thought and study which it merits. Most of such studies have come from England. Campbell and Warner[7] reported that rheumatic fever was the most crippling affection of the poor. Glover[12] believed that no disease had a more clear-cut social incidence than rheumatic fever and estimated the occurrence of acute rheumatism as 20 or even 30 times as great in the poor as in the well-to-do. Miller[29] found that, although the frequency did not seem to follow absolutely the variations in degree of poverty, "yet nothing is more certain than that it is a disease of the poorer classes." Coombs[8] noted that it was quite certainly a rare disease among the well-to-do. Among 1,000 children from the Out-Patient Department of King's College Hospital, London, England, the incidence of those showing evidence of acute rheumatism was 13.1 per cent whereas, among 700 children from private practice, the incidence was only 0.7 per cent,[27] a ratio of 19 to 1. Paul[31] found a less striking, though distinct, association with poverty in the studies he made in New Haven, Conn. It seems probable that less marked differences in social-economic conditions were present in the New Haven groups. There are, of course, a number of factors which might be operative in the different social environments. To the writer, it appears unlikely that hereditary influences would be responsible for striking differences in "social incidence." Although little is known with regard to the incidence of hemolytic streptococcic infections in various social categories, it also appears extremely improbable that the organism would exhibit the striking social selectivity observed in rheumatic fever.

One variable factor intimately associated with social environment is undoubtedly nutritional. It would appear that deficiency of vitamin C and the associated bioflavonoids* might be important host factors. Briefly reviewed, the evidences indicating this are as follows:

(1) Experimental scurvy in the guinea pig, combined with streptococcal infection, occasions rheumaticlike lesions while, with the infection alone, no cardiovascular or articular lesions occur.

(2) The remarkable clinical "experiment" reported by Glazebrook *et al.* strongly implicates vitamin-C deficiency as an important predisposing factor in the pathogenesis of the initial occurrence of rheumatic fever.

(3) The particular cytologic alteration characteristic of the "rheumatic" lesion (*i.e.*, the Anitschkow cell) is favored by the presence of vitamin-C deficiency and may be seen in uncomplicated scurvy.

(4) The hemorrhagic manifestations of rheumatic fever (particularly epistaxis) have been greatly reduced since the importance of vitamin C in general nutrition and, specifically, in rheumatic fever, has been recognized.

(5) We have reported that the administration of vitamin C and bioflavonoids exerts a favorable influence on the course of rheumatic fever. These studies are admittedly not fully conclusive in that the numbers of cases studied is relatively small and the periods of observation are short. It is reported that bioflavonoids have reduced the incidence and severity of epistaxis in patients with rheumatic fever.[21]

(6) Vitamin C alone, when administered in massive dosage, has been said to possess antirheumatic activity.[24]

If vitamin C and associated bioflavonoids play a role in the biologic defense mechanism concerned in the genesis of rheumatic fever, how do they act? These substances are importantly concerned in the economy of connective tissue ground substance. Vitamin C is known to be essential for the elaboration of ground substance[32] as well as of the fibrillar elements of connective tissue.[49] Bunting and White[6] observed that acid mucopolysaccharides are more abundant in the connective tissue of wounds of partially scorbutic animals than normal ones. Pirani[33] observed that vitamin-C deficiency *per se* occasions some depolymerization of the connective tissue ground substance which is reflected by an increase in the mucopolysaccharide content of the blood. An increase in the polysaccharide components of the blood plasma has also been observed in rheumatic fever.[45] The intimate locus of the rheumatic injury is in the ground substance. This lesion may be related to the release of a depolymerizing agent such as hyaluronidase acting upon a tissue rendered particularly vulnerable by deficiencies of ascorbic acid and bioflavonoids. Repeated injections of large doses of hyaluronidase produce lesions in the heart and joints with a limited resemblance to those of rheumatic fever.[2] Hartmann and Matejevic[16] noted that the drugs most effective in the management of rheumatic fever are hyaluronidase inhibitors. The general question of hyaluronidase inhibition has been recently reviewed by Mathews and Dorfman.[25] Bio-

* These two factors are properly considered together; they are commonly associated in nature. Among other activities, bioflavonoids exert a stabilizing influence on ascorbic acid. (See Gustav J. Martin.[23])

flavonoids have been shown to possess antihyaluronidase activity which is potentiated by ascorbic acid.[23]

The experimental lesions resulting from the administration of desoxycorticosterone and excess sodium chloride are typified by marked swelling of the ground substance of connective tissue (in this sense, analogous to the injury occurring in rheumatic fever and possibly involving hyaluronidase activity). It is noteworthy that this desoxycorticosterone-salt injury can be essentially inhibited by the administration of the bioflavonoid, hesperidin methyl chalcone.

It would appear, then, that there are multiple evidences which constitute a substantial basis for the use of ascorbic acid and bioflavonoids in the prophylaxis and management of rheumatic fever.

Summary

Observations are recorded indicating that vitamin C and bioflavonoids may play a significant role in the biological defensive mechanisms concerned in the genesis of rheumatic fever. Heart valve and myocardial lesions resembling those of rheumatic fever develop in guinea pigs subjected to the combined influence of scurvy and hemolytic streptococcal infection. There is substantial clinical evidence that the tissue reserves of vitamin C are commonly depleted at the time of onset of rheumatic fever. An important clinical study carried out in the Royal Navy (British) in World War II affords direct evidence that vitamin-C deficiency, in the presence of hemolytic streptococcal infection, disposes to the development of rheumatic fever.

A study of early lesions of rheumatic fever indicates that the initial reaction to the rheumatic injury is one involving a swelling of the ground substance (mucopolysaccharide) of connective tissues, particularly that of the heart valves, heart muscle, and joints. Hyaluronic acid is a major component of these tissues. The *beta* hemolytic streptococcus is the only bacterium clearly implicated in the pathogenesis of rheumatic fever and is unique among bacteria in possessing a capsule composed of hyaluronic acid. It seems likely that the development of rheumatic fever involves some injurious mechanism causing depolymerization of the hyaluronic acid components of connective tissue. It may be that hyaluronidase, elaborated as a defensive reaction to hemolytic streptococcal infection, may act not only on the bacterial capsule but on the chemically related ground substance of connective tissue or, possibly, an antigenic substance derived from the capsulated hemolytic streptococcus may become incorporated in the ground substance of connective tissue and, on interacting with antibody, may release a depolymerizing agent such as hyaluronidase.

Our studies, as well as those of others, indicate that vitamin C and bioflavonoids are importantly concerned in the economy of the connective tissue ground substance. Vitamin C is essential for the elaboration of the ground substance as well as the fibrillar element of connective tissue. Vitamin-C deficiency *per se* occasions some depolymerization of the connective tissue ground substance. Thus, the existence of a deficiency of vitamin C and associated bioflavonoids would appear to render these tissues particularly vulnerable to injury.

A number of studies, including our own, indicate that bioflavonoids serve in some way to stabilize the connective tissue ground substance. Several of the substances have been shown to possess antihyaluronidase activity. This activity is potentiated by ascorbic acid. We have found that the flavonoid, hesperidin methyl chalcone, administered *per os* to guinea pigs, will diminish the spreading reaction induced by intracutaneous hyaluronidase.

Repeated intravenous injections of hyaluronidase in rabbits cause foci of mucinous edema in the interstitial tissue of the heart that bear limited resemblance to the rheumatic lesions.

Our studies indicate that the experimental desoxycorticosterone-salt injury resulting in interstitial myocarditis and periarteritis is due to a swelling of the mucopolysaccharides of the interstitial tissues and of the vascular wall. We have found that administration of the bioflavonoid, hesperidin methyl chalcone, will protect the animal from such injury. Limited direct evidence is presented that ascorbic acid and bioflavonoids possess therapeutic value in the management of cases of rheumatic fever.

The importance of infection with the *beta* hemolytic streptococcus in the etiology and pathogenesis of rheumatic fever is fully recognized. Host factors concerned in resistance, however, should not be ignored. It appears that there is much evidence which constitutes a sound basis for the use of ascorbic acid and bioflavonoids in the prophylaxis and treatment of rheumatic fever.

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DECIDUAL BLEEDING IN PREGNANCY

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Varying degrees of decidual bleeding take place into the maternal portion of the placenta during pregnancy. Physiologic or implantation bleeding is slight and usually occurs soon after the first missed menstrual period, as the "placental sign." Later on, moderate antepartum bleeding may occur which is regarded clinically as a threatened abortion. When the bleeding becomes excessive, the abortion is usually considered as inevitable. Under such circumstances, physiologic, decidual hemorrhage has assumed pathologic proportions.

Decidual hemorrhage was described by Cruveilhier[2] in 1829 as hemorrhage into the "inter-utero-placental" region, and such hemorrhage was regarded by him as a cause of abortion. For more than a century, a controversy has continued as to whether the decidual hemorrhage is a primary or a secondary effect of the spontaneous abortion sequence. The two schools of thought have recently been reviewed by Eastman,[3] who concluded: "Until recently, it was generally accepted that the decidual hemorrhage was the primary lesion and that the death of the embryo was secondary. However, modern studies have demonstrated that the reverse is usually true. The death of the embryo is primary and the decidual hemorrhage is secondary." Our observations, made on 1334 decidual specimens, do not support these conclusions but rather confirm the older views, as shown by data in TABLE 1. This table shows data from 1334 abortion specimens containing decidua. Decidual hemorrhage was present in 61 per cent of them. Moreover, the incidence of decidual hemorrhage was nearly 60 per cent in cases diagnosed clinically as a threatened abortion on their admission to the hospital and subsequently becoming complete; whereas cases clinically diagnosed as a missed abortion, in which the fetus usually dies *in utero* and is later expelled in a macerated condition, had an incidence of decidual hemorrhage in only 30 per cent of the cases.

Few histologic studies have been made of the decidua *in utero* in early pregnancy, and the work of Rutherford[13] in 1942 is outstanding. Rutherford obtained decidual biopsies on 100 consecutive patients with threatened abortion and found "decidual apoplexy," consisting of hemorrhage, necrosis, cellular infiltration, and vascular thrombosis, similar to that shown in FIGURE 1. He attributed these pathologic changes to progesterone deficiency and therefore advocated corrective endocrine therapy. Javert and Stander,[5] in the same year, postulated that deficiency in vitamins C and K might cause the decidual bleeding. They offered this theory as one of the factors in the pathogenesis of spontaneous abortion, basing it on their laboratory evidence of hypovitaminosis C and K. Meanwhile, pathologic studies of the relationship of vitamin C deficiency to decidual hemorrhage were initiated but were postponed because of World War II. Later, these studies were resumed, and results have been recently published[8] and will also be referred to in the present article. TABLE

TABLE 1

CONDITIONS FOUND IN THE DECIDUA OF 1334 ABORTION SPECIMENS AS COMPARED WITH 361 CONTROL SPECIMENS*

	Spontaneous number	Abortion Per cent	Control group*	
			Number	Per cent
Hemorrhage	810	60.7	31	8.6
Degeneration	680	50.9	75	20.7
Infection	572	42.8	13	3.6
Normal	83	6.2	268	71.5

* Therapeutic and unintentional abortion specimens.

2 reveals decidual hemorrhage in 67 per cent of the specimens when the mothers were deficient in vitamin C, as determined by blood studies. Biochemical studies have been made on the decidua with inconclusive results. Further attempts should be made on this tissue, since it is the only maternal portion of the placenta.

Barnes[1] made assays of vitamin C of the placenta in 1947, and Holzaepfel and Barnes[4] studied placental metabolism of vitamin C. They found the greatest concentration of C in the syncytial layer of the villi, which are of fetal origin. Other fetal tissues have high values, including the cord blood, as shown by Javert and Stander,[5] in comparison with the maternal blood concentration at term. Power[10] reported 13 patients with degenerated decidua, in 1948. He regarded this as a cause of the decidual bleeding, and questioned the value of endocrine therapy. Javert and Finn[6] also found frequent evidence of decidual hemorrhage and degeneration in a preliminary report of 500 cases of spontaneous abortion, which has been confirmed by a larger series of 1334 cases, as shown in TABLE 1. They suggested preventive measures consisting of a diet rich in citrus fruits and the use of vitamin C and K supplements.

Ramsey[11] studied the pregnant rhesus monkey and found that the spiral arterioles of the myometrium and endometrium uncoiled like a rubber hose as the uterus increased in size, whereas the veins, usually straight and distended, became very stretched. Continuity of these veins is often ruptured during pregnancy, producing antepartum bleeding. A ruptured decidual vein, of an actual case, is shown in FIGURE 2. The veins also rupture with the expulsion

TABLE 2

DECIDUAL HEMORRHAGE IN 100 CASES OF SPONTANEOUS ABORTION CORRELATED WITH MATERNAL BLOOD PLASMA VITAMIN C DEFICIENCY*

Maternal blood plasma	No. of cases	Decidual hemorrhage	
		Number	Per cent
Vitamin C deficiency average value 0.22 mg. %	45	30	66.6
Vitamin C sufficiency average value 0.95 mg. %	55	22	40.0
Total	100	52	52.0

* Values below 0.50 mg./per cent.

FIGURE 1. Decidual hemorrhage in a spontaneous abortion specimen. Note the vasodilatation and the extravasation of blood into the stroma.

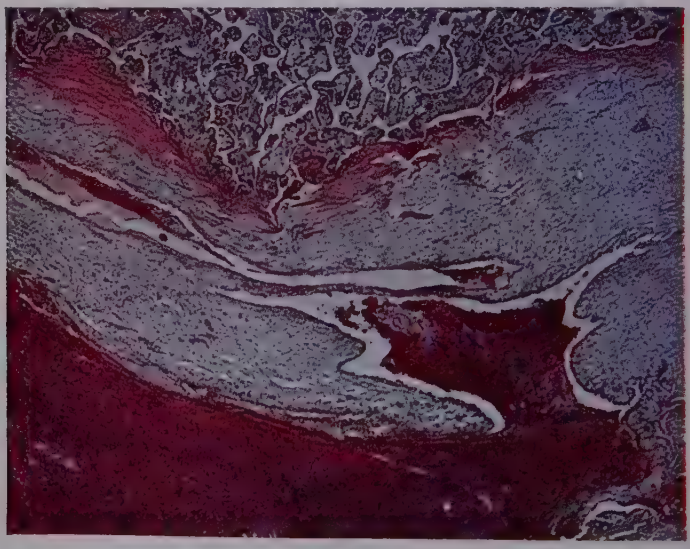

FIGURE 2. Photomicrograph, low power, showing a ruptured decidual vein that bled antepartum, before the abortion occurred. Note retroplacental clot.

FIGURE 3. The maternal, decidual surface of a delivered, normal, term placenta. Plastic toothpicks have been inserted into the torn ends of the decidual vessels which formerly connected the placental intervillous spaces with the uterine veins and the maternal circulation.

of the placenta in the third stage of labor. Examination of the decidual plate reveals numerous venous openings, as indicated in FIGURE 3. These represent potential sites of rupture of the decidual veins, often seen in partial, complete, marginal, and concealed premature separation of the placenta. Moreover, the increase in maternal blood volume serves to overdistend the veins, as does pelvic congestion due to portal or arterial hypertension, toxemia of pregnancy, hot baths, heating pads, coitus to orgasm, and fright. Here, then, are two normal physical factors which may cause decidual hemorrhage: (1) the vasodilatation, engorgement, and stretching of the veins to the breaking point; (2) occurrence and continuation of the hemorrhage when the bleeding and clotting mechanisms are not in balance. Another important biologic factor to contend with is trophoblastic erosion, invasion, and perforation of the decidual veins, shown in FIGURES 4 and 5.

Placentation and the development of the placenta can be compared to the growing onion, sending forth concentric rings, as shown in FIGURE 6. The

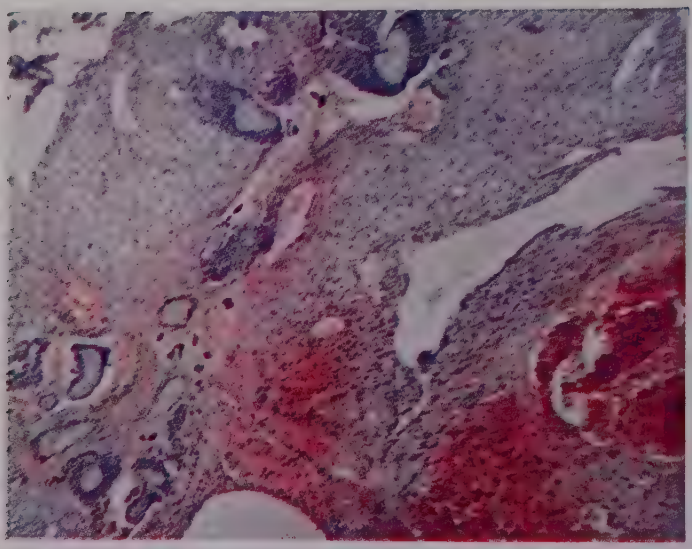

FIGURE 4. Photomicrograph, medium power, showing trophoblastic invasion and perforation of a decidual vessel and the resulting stromal hemorrhage seen nearby.

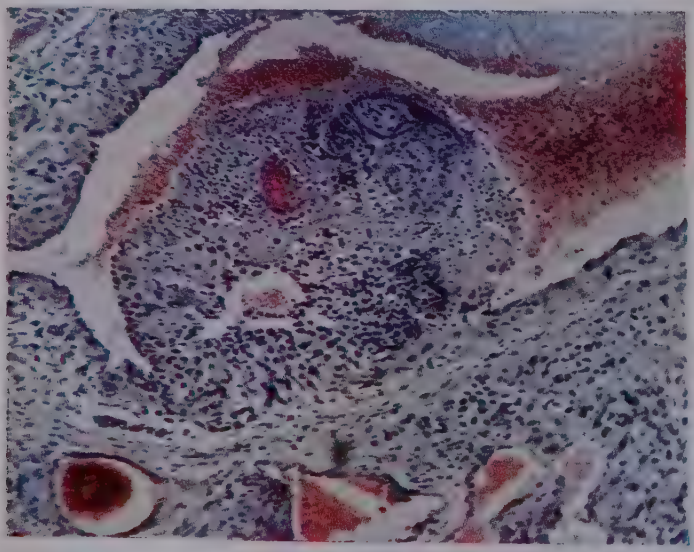

FIGURE 5. Another example of invasion of decidual vein by trophoblast.

concomitant, circular, trophoblastic erosion and invasion of the decidua basalis, the decidual sinuses, and the underlying myometrium in order to anchor the placenta, provide the primary or basic mechanism for decidual hemorrhage. The bleeding and clotting mechanisms and their states of balance and imbalance are secondary or associated factors. Some of the known primary and secondary factors constituting the mechanism of decidual hemorrhage are shown in FIGURE 7.

The increased requirements of pregnancy cause a physiologic deficiency of

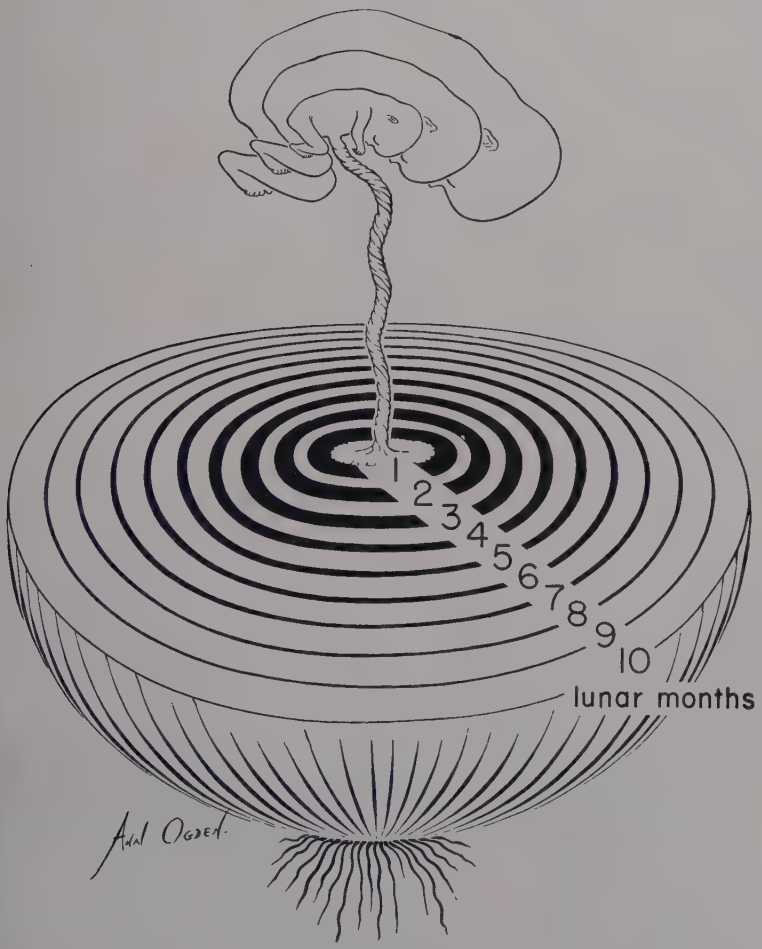

FIGURE 6. The placenta enlarges and develops in concentric rings like an onion. Concomitantly, a circular, rophoblastic erosion and invasion of the decidua basalis causes decidual hemorrhage.

minerals, vitamins, and other compounds. Some of these have to do with bleeding and clotting of blood. It is our purpose now to see how sufficiency of these factors control normal, physiologic, decidual hemorrhage; and how deficiency produces abnormal, excessive, and pathologic decidual hemorrhage. Obviously, the target area for preventive, medical therapy is the decidua basalis.

The diagram shown in FIGURE 7 summarizes some of the probable maternal factors which have to do with decidual bleeding and clotting mechanism in pregnancy. They include uterine contractions, stretching of the veins, trophoblastic invasion of the decidual veins, vitamin C, P, and K deficiency, afibrinoginemia, hormone deficiency, surgical removal of corpus luteum, calcium deficiency, vasodilation due to increased maternal blood volume, arterial and/or portal hypertension and orgasm. These may be contributing causes for the decidual hemorrhage which is so frequently found in most of the spontaneous abortion specimens. The effects of afibrinoginemia, hypoprothrombinemia, vitamin K deficiency, hormone and calcium deficiency or blood coagulation are frequently discussed in the current literature. The time-honored condition of scurvy is seldom mentioned. It will be discussed now at length, since it is

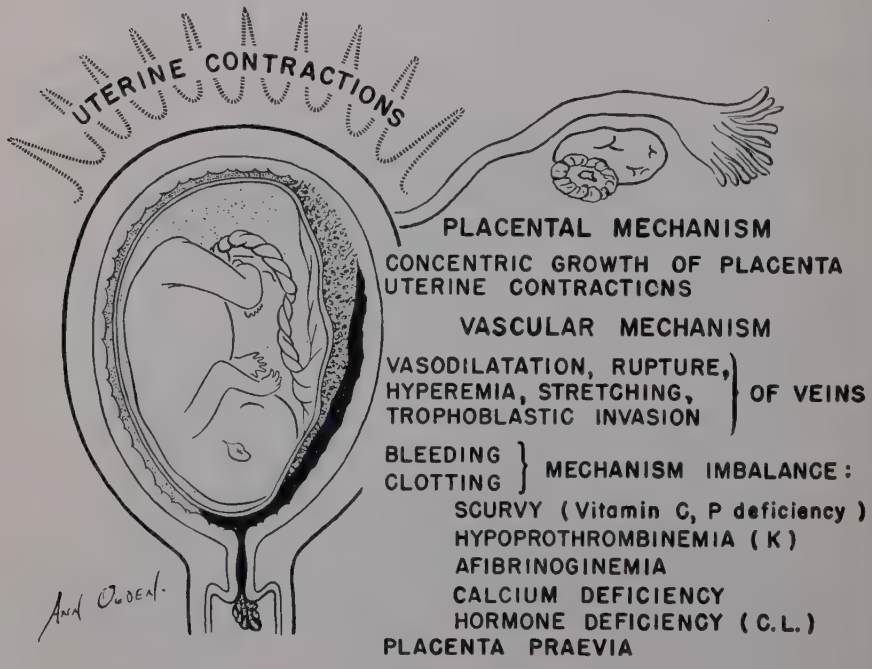

FIGURE 7. Mechanism of decidual hemorrhage includes a number of factors.

regarded by the author as having a prominent etiologic role in the production of the decidual hemorrhage, along with several other factors shown in FIGURE 7. It becomes apparent that decidual hemorrhage is governed by many interrelated biologic, physiologic, pathologic, chemical, physical, and obstetrical factors. The role of vitamin C and P deficiency in producing decidual hemorrhage will now be discussed.

During pregnancy, 420 blood plasma vitamin C determinations were made on 300 obstetrical patients and a graph of the values is shown in FIGURE 8.

The plasma vitamin C concentration decreases in normal pregnancy from an average nonpregnant value of 1.0 mg. per 100 cc. to 0.29 mg./cc. at term, when the fetal cord blood has a value of 1.4 mg. per cent. By the 16th week of pregnancy, the average value had decreased to 0.48 mg. per cent, as shown in FIGURE 8. By this time, the spontaneous abortions are in progress or will have taken place. Recent attempts to reduplicate these data have not been successful, probably because of the widespread publicity as to the value of orange juice in preventing abortion.

The weight of the fetal protoplasmic mass increases more than 1000 times from 0.0+ mg. to 500 gm. during the first 22 weeks of its development. Thereafter, in the remaining 18 weeks of gestation it increases only sevenfold, to 3500 gm. Therefore, it is during the first half of gestation that the vitamin C requirement is the greatest. Most spontaneous abortions occur in the 3rd or 4th month, and 87 such patients, or 54 per cent, had values below 0.5 mg. per

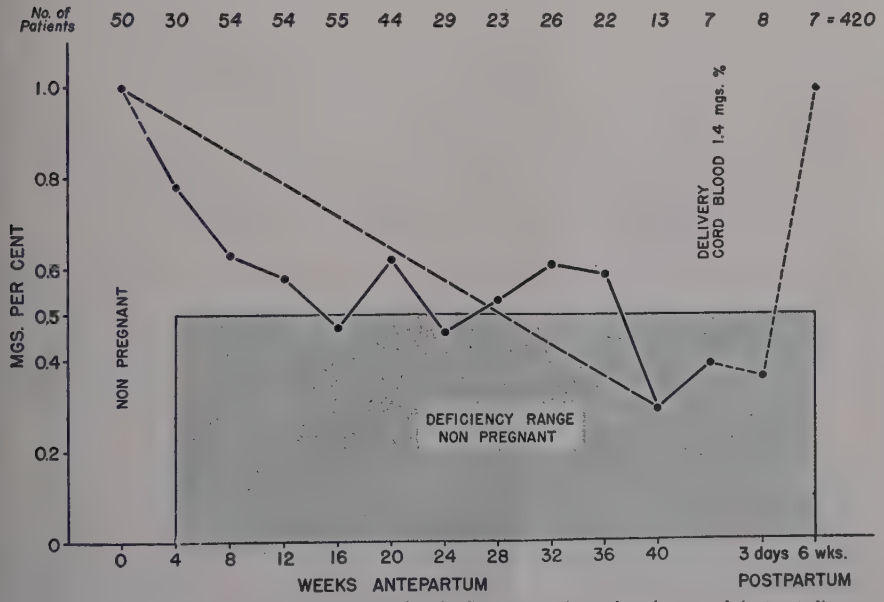

FIGURE 8. Average, maternal blood plasma vitamin C concentration values in normal (untreated) pregnancy. At delivery, the fetal cord blood value is much higher.

TABLE 3

MATERNAL BLOOD PLASMA VITAMIN C CONCENTRATION IN 354 UNTREATED PATIENTS IN EARLY PREGNANCY, COMPARED WITH TREATED PATIENTS AND OTHER CASES OF SPONTANEOUS ABORTION

	No. of cases	Maternal plasma vitamin C concentration		
		Average value in mg./%	Deficiency*	
			No.	%
Untreated pregnancy (8–22 weeks)	237	0.58	110	46.6
Treated pregnancy†	25	1.26	2	8.0
Spontaneous abortion (untreated)	161	0.47	87‡	54.0

* Values below 0.50 mg./per cent.
† Total daily intake of 500 mg. of vitamin C 150 mg. hesperidin.
‡ 20 cases had values of 0.00 mg. per cent.

cent, as indicated in TABLE 3. Against this experience, other data, shown in TABLE 2, correlate the vitamin C values with decidual hemorrhage. It can be seen that 67 per cent of the patients below 0.5 mg. per cent had decidual hemorrhage, whereas only 40 per cent had decidual hemorrhage when the vitamin C values were normal. Obviously, there are other factors governing decidual hemorrhage, as indicated in FIGURE 7.

TABLE 4 gives the results of forced vitamin C intake during the first half of pregnancy in 25 normal obstetrical patients. Before the therapy, the average value was 0.42 mg. per cent, clearly in the deficiency range. It rose to 1.26 mg. per cent after a daily intake of 500 mg. of vitamin C, obtained as follows: diet rich in citrus fruits provided 350 mg. and vitamin supplement of hesperidin C* supplied 150 mg. of "C" and 150 mg. of "P." Meanwhile, during the treatment, the incidence of plasma vitamin C deficiency decreased from 48 per cent to 8 per cent. This evidence would suggest that the daily requirement of vitamin C during pregnancy is far in excess of the 100 mg. per day stated by the National Research Council. Thysell found that this amount was necessary to produce and maintain a blood level of 1.0 mg. per cent in nonpregnant individuals.

Many of the obstetrical patients develop idiopathic bleeding from the nose, gums, skin ecchymoses, and anus, with no specific pathology to account for the hemorrhage during pregnancy, and 47 per cent of these patients have a vitamin C deficiency as shown in TABLE 3. This history was often encountered in those having spontaneous abortion. Blood studies on such patients often revealed a low blood plasma vitamin C concentration, ranging from 0.00 mg. per cent to 0.35 mg. per cent. Forced vitamin C therapy raised the blood values to normal and controlled this bleeding tendency in the majority of the patients. A typical case is reported herewith:

Case No. 1. Mrs. P. K., age 25, primigravida, was first seen in the sixth week of pregnancy, at which time she was experiencing severe nausea and a loss of six pounds in weight. The physical examination was not remarkable except

* Hesperidin-C supplied through the Medical Research Department, The National Drug Company, Philadelphia, Pa.

Table 4

Plasma Vitamin C Concentration of 25 Normal Obstetrical Patients in Early Pregnancy, Before and During Forced Vitamin C Therapy*

	No. of cases	Plasma vitamin C concentration		
		Average values in mg. %	Deficiency	
			No.	%
Before therapy	25	0.42	12	48.0
During therapy	25	1.26	2	8.0

* High citrus diet daily intake—350 mg.
Hesperidin C 50 mg. t.i.d.— 150 mg.
Total daily intake 500 mg.

for a third-degree retroversion uteri, which was easily corrected. Prenatal instructions, including a high citrus diet, were advised. The nausea abated, and the patient regained several pounds of weight during the next six to eight weeks. Soon thereafter, the gums began to bleed and her dentist "scaled" her teeth and told her that she had pregnancy gingivitis. Nevertheless, the gums continued to bleed. In the 19th week of gestation, an idiopathic swelling occurred in the left foot. At the same time, slight nosebleeds occurred. At this time, she was questioned as to easy skin bruising, which she said had been present "all my life." The menses had been regular, but profuse with a heavy flow. She admitted that she had not been drinking orange juice as originally advised. A digestion plasma vitamin C test revealed a level of only 0.3 mg. per cent. She was placed on Hesperidin-C (50 mg. C and 50 mg. hesperidin) three times a day and large amounts of orange juice. Four weeks later, the blood value was 1.3 mg. per cent, showing that the C deficiency was corrected to a nonpregnant value despite the increased needs of the gestation. There were no further nosebleeds, and the bleeding from the gums ceased. The pregnancy continued uneventfully to term and she was delivered of a 3600 gm. infant.

The occurrence of hemorrhage in the decidua or maternal portion of the placenta may be a manifestation of scurvy. The decidual blood vessels are composed of endothelial cells held together by intercellular cement. They usually have no fibromuscular structures and, therefore, are unable to contract. Vasodilatation and engorgement cause them to become greatly distended, as seen in many of our abortion specimens. Stefanini states that the "cement contains hyaluronic acid and that vitamin C is necessary for its synthesis." A defect in this "cementing substance" may be responsible for the spontaneous onset of bleeding of distended decidual veins.

Vitamin P, or citrin, was isolated by Rusznyák and Szent-Györgyi[12] in 1936, the last-named author also having discovered vitamin C in 1928. This compound has received considerable attention in the literature because its properties resemble those of vitamin C. "Citrin" contains hesperidin and eriodictyol. The active principle is hesperidin. Vitamin P, a constituent of orange peel, flowering buckwheat, *etc.*, is not as readily available as C in the diet, so that a supplement may be necessary. Such a compound is commercially available as Hesperidin-C.

The decidua was normal in only 6 per cent of the 1334 spontaneous abortion

specimens, as shown in TABLE 1. Decidual hemorrhage was found in 61 per cent, while the control group of 361 therapeutic and unintentional abortions specimens had decidual hemorrhage in only 9 per cent, as revealed in TABLE 1. The other abnormal conditions found in the decidua are given in TABLE 1, including degeneration and infection, which probably are results of the abortion process.

Blood determinations were obtained in another group of 354 patients at the time of the first antenatal visit, at the time of hospital admission for threatened abortion and at the time of admission for spontaneous abortion. Approximately 45 per cent of the patients in these three groups had a vitamin C deficiency. These patients had received little or no dietary instructions or vitamin C therapy prior to being studied. The average plasma C value was 0.58 mg. per cent for normal pregnancy, and it is slightly lower for patients with threatened and spontaneous abortion, who had decidual hemorrhage, each having values of 0.4 mg. per cent. The group without decidual hemorrhage had a higher value of 0.76 mg. per cent. The percentage incidence of vitamin C deficiency was 56.6 per cent in the group with decidual hemorrhage and only 31 per cent when there was no hemorrhage. These figures approximate the pathologic incidence of decidual hemorrhage when there is maternal deficiency, as shown in TABLE 2.

The time of occurrence of vitamin C deficiency in pregnancy ending in abortion is unknown. The curve shown in FIGURE 8, however, indicates a marked drop by the third month, when most abortions occur. When a deficiency has existed before pregnancy, the tissue unsaturation is even greater, which predisposes to spontaneous abortion. It can be seen from data in TABLE 5 that 29 per cent of nonpregnant, gynecologic patients without menorrhagia and 31 per cent of the 50 nonpregnant obstetrical patients were deficient in C. The average incidence becomes 45 per cent in normal pregnancy. This difference of 15 to 17 per cent represents those additional patients who have developed a deficiency because of the increased requirements of pregnancy. Incidentally, the author has seen some cases of idiopathic menorrhagia respond to forced vitamin C, K, and hesperidin therapy.

A distinct correlation was found between vitamin C deficiency and decidual hemorrhage, as revealed in TABLE 2. When there was a maternal deficiency in

TABLE 5

BLOOD PLASMA VITAMIN C CONCENTRATION IN NONPREGNANT PATIENTS WITH AND WITHOUT MENORRHAGIA

		Plasma vitamin C concentration		
	No. of cases	Average values in mg. /%	Deficiency	
			No.	%
With menorrhagia	28	0.48	16	57.1
Without menorrhagia	75	0.79	22	29.3
Total	103		38	36.8

C, with an average value of 0.22 mg. per cent, decidual hemorrhage was found in 66 per cent of the respective abortion specimens. Conversely, when there was a sufficiency of vitamin C, decidual hemorrhage was observed in 40 per cent of the specimens, thus signifying that there are other causes of such bleeding, as outlined in FIGURE 7.

The observations made in the laboratory on the relationship of vitamin C to decidual hemorrhage and spontaneous abortion have been supplemented by a clinical study of patients with repeated or habitual spontaneous abortion. By definition, such patients have had three or more consecutive spontaneous abortions and represent an excellent clinical proving ground for investigation of concepts developed in the laboratory.

The writer's personal experience with 100 of these patients, observed in 145 pregnancies cared for during an 18-year period, could be duplicated only in the lifetime of four or five busy obstetricians. These patients had lost 420 pregnancies by miscarriage before being cared for by the author.

The program of therapy, described elsewhere,[7] included a diet rich in citrus fruits providing 350 mg. of vitamin C and a Hesperidin-C supplement of 50 mg. three times daily, for a total of 150 mg. of vitamin C and 150 mg. of hesperidin daily. The total intake of C averaged 500 mg. per day. The total premature and full-term outcome after treatment was 91 per cent for the entire group of 100 delivered patients. The few that aborted had decidual hemorrhage in only 5 per cent of the specimens.

Following the successful or unsuccessful termination of the first, or initial, pregnancy on such therapy, 18 of these habitual abortion patients undertook further pregnancies, 45 in all. The repeat, successful, premature and full-term delivery incidence has been 81 per cent. Nine had abortions, one was therapeutic, and five occurred to two patients who purposely and willfully omitted the specified treatment since neither was ready for another child. If these six willful abortions are excluded, then a corrected incidence of repeated success would be in the neighborhood of 90 per cent. Once these patients succeed, they continue to be successful time and time again.

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Discussion of the Paper

Doctor Blumberg: Do you make any distinction between the use of citrus fruits for this high vitamin C and the use of plain ascorbic acid? Do you feel that the use of citrus fruit is superior to the plain ascorbic acid in addition to the hesperidin that you did use?

Doctor Javert: We do not consider that there is a difference between the efficacy of the two and yet, as a clinician, I am not qualified to discuss the merits of either therapy. We do prefer, however, a large amount of the natural vitamin, from whatever source it comes, for several reasons. For one thing, we took away mineral oil from our patients and found that large amounts of vitamin C, particularly in orange juice or fruit slices, were very effective in combating the problem of constipation.

Doctor Blumberg: Do you feel that the hesperidin component has aided materially in your treatment, or do you think that vitamin C is the main factor?

Doctor Javert: Prior to 1950, we used the straight ascorbic acid and we averaged, with some 50 patients, an 87 per cent successful outcome. Since changing to the Hesperidin-C, we are pushing much closer to 95 per cent in another 50 patients.

Doctor Lewis Levin (*Columbia University, New York, N. Y.*): How about vitamin C in anemia of pregnancy?

Doctor Javert: Vitamin C has not helped at all with the anemia of pregnancy. Iron supplements are needed to correct this condition.

THE MANAGEMENT OF HABITUAL ABORTION*

By Robert B. Greenblatt
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Habitual abortion may be the result of many factors, including hormonal imbalance, defective germ plasm, blighted ovum, poor implantation, cervical incompetence, and uterine anomalies. The seminal factor, too, may not be ignored. Consequently, no single regimen of therapy can be applied to all cases. One rather frequent finding in habitual aborters is the presence of ecchymotic areas resulting from minor bruises. All women in the author's series with a history of spontaneous abortions were tested for evidence of abnormal capillary fragility (FIGURE 1). Positive tests were obtained in over 80 per cent, a much higher incidence than that found in a control group.[1, 2] Hence, an effort was made to lessen capillary fragility and to correlate the correction of this factor with the incidence of subsequent abortions.[3]

A concept had been offered that a hemorrhagic diasthesis, as manifested by nasal, gingival, rectal, and dermal bleeding, in addition to the vaginal bleeding of threatened abortion may reflect a vitamin C and P deficiency. Many of these patients had low values of ascorbic acid in the blood plasma, and others were also low in prothrombin concentration.[7] It seemed significant that abortions occurred most commonly in the second and third months of gestation when vitamin C and vitamin K levels were found to be low.[8] Low capillary resistance in patients with various forms of nutritional deficiency was found to improve following the administration of a flavonoid, hesperidin, or certain vitamin P-containing extracts, while treatment with ascorbic acid and other vitamins did not influence the condition.[9]

Since 1946, hesperidin as vitamin P and ascorbic acid as vitamin C were used in our habitual aborters to treat capillary fragility following the work of Szent-Györgyi and others.[4, 5] Bed rest was ordered when uterine cramps or spotting occurred, especially in those women with proved cervical inadequacy. Thyroid medication was given to patients with high cholesterol or low protein-bound iodine values. Antianemia agents were administered whenever indicated, and multivitamins were employed routinely.

Increasing cornification of the vaginal smears has been used as an index of impending abortion.[10, 11] A most valuable clue in our hands has been the appearance of fern in the cervical mucus during pregnancy[12-14] (FIGURES 2a and 2b). Whenever such fern appeared in the cervical mucus, anterior pituitary-like hormone (A.P.L.) was given. The dosage used was 2000 to 4000 units daily, gradually increasing the interval between injections to 7 to 14 days. Progesterone was frequently added to fortify the chorionic gonadotropin therapy until the fern disappeared and the cervical mucus again yielded the typical cellular pregnancy response (FIGURES 3a and 3b). In many instances, therapy

* Grateful acknowledgment is made to the following pharmaceutical companies for supplies of medications used in this study: National Drug Company, Philadelphia, Pa. for Hesperidin C; Ciba Pharmaceutical Products, Inc., Summit, N. J., for Lutocylin; Ayerst, McKenna and Harrison, Ltd., for A.P.L.

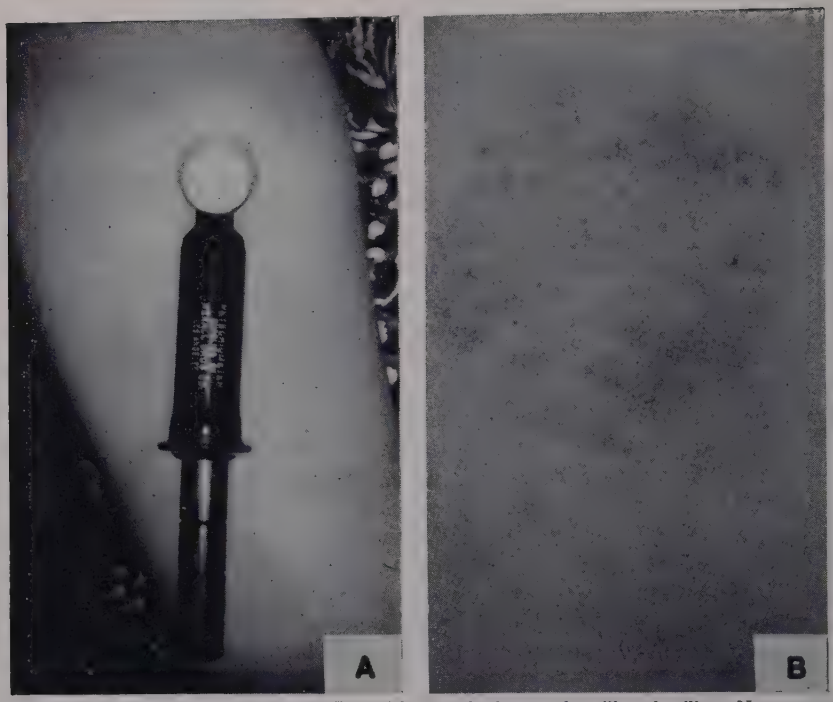

FIGURE 1. A. Petechiometer in place. B. Positive test for increased capillary fragility. Note numerous minute petechial hemorrhages. (Reproduced from R. B. Greenblatt. 1953. Obstet. and Gynecol. 2: 530.)

was carried on empirically. When abortion threatened, large doses of progesterone (100 mg.) with relatively small doses of estrogens and/or chorionic gonadotropin were given.

Stilbestrol, as advised by the Smiths,[15] was given a short trial several years ago, but was abandoned as contraphysiological and contrary to our way of reasoning. Much controversy still remains as to whether stilbestrol improves progesterone metabolism. It is our opinion that one of the hormonal causes for abortion is probably the unopposed action of estrogen, as manifested by the appearance of the palm arborization (fern) phenomenon and the cornified vaginal epithelium, and we feel that this action is the result of inadequate endogenous progesterone production. It would appear, if this reasoning is correct, that massive doses of estrogens would be contraindicated. Measures to improve or fortify progesterone production, *i.e.*, stimulation and sustaining of corpus luteum activity during the first few months of pregnancy, would seem a more sensible approach. To this end, chorionic gonadotropin may be employed. The addition of progesterone, to bolster the effectiveness of chorionic gonadotropin, should also serve to buffer estrogen activity. The use of progesterone, rather than stilbestrol, has also been strongly advocated by Alder,[16] Bishop and Richards,[17] Swyer and Daley,[18] Greenhill,[19] Koff and Tulsky,[20] and others.

FIGURE 2. A. Some fern formation in cervical mucus during pregnancy in patient threatening to abort. B. Note fern formation along with massive cellular response in habitual aborter.

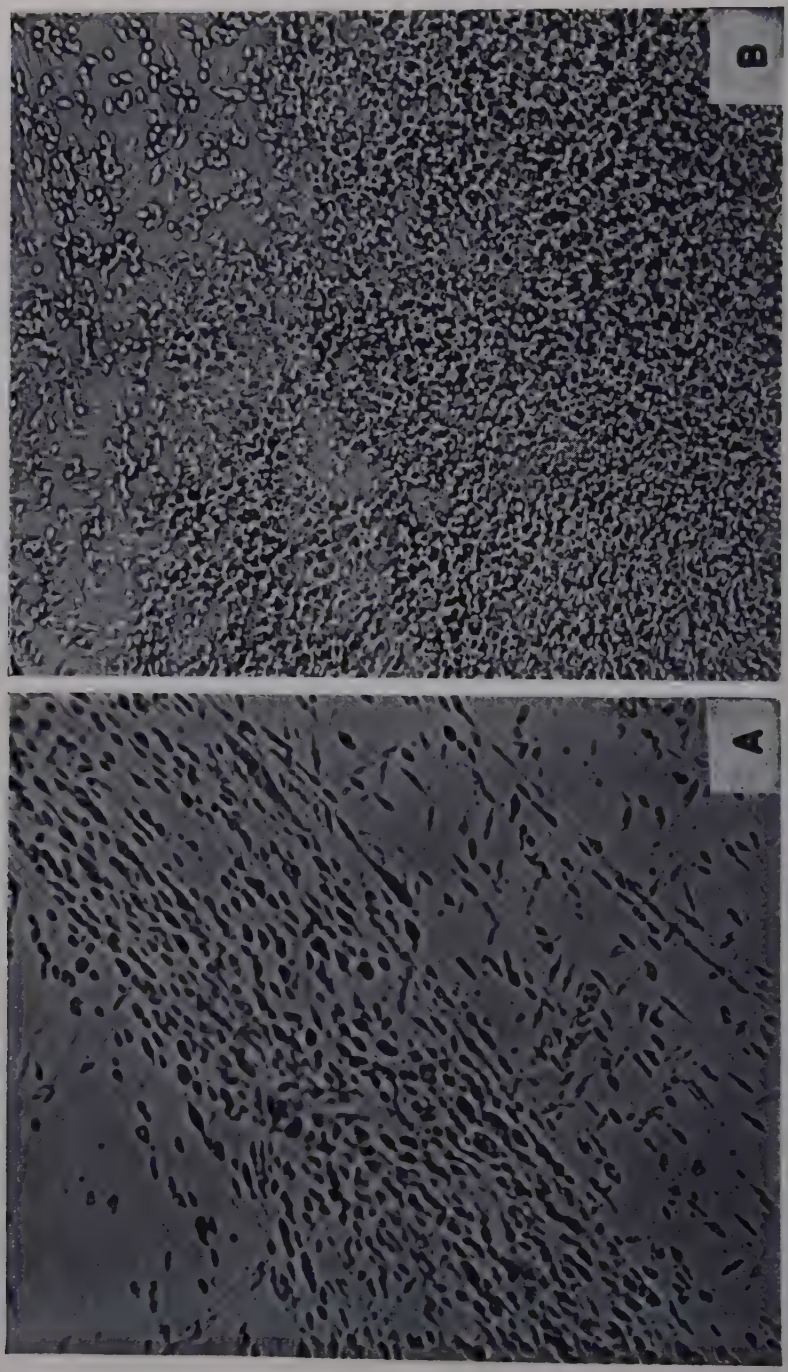

FIGURE 3. A. Note absence of fern formation following treatment. B. Note absence of fern formation and massive cellular response following therapy.

TABLE 1

COMPARATIVE RESULTS OBTAINED IN TREATMENT OF HABITUAL ABORTION WITH A VARIETY OF METHODS

Author	Agent	Salvage	Criterion (No. of abortions)
Davis and Fugo	Stilbestrol	45%	2 or more
	Progesterone	66%	2 or more
Swyer and Daley	Control	75%	2 or more
	Progesterone	80%	2 or more
Smiths	Stilbestrol	73%	3 or more
Bishop	Progesterone	72%	3 or more
Watson and Tew	Vitamin E	73%	3 or more
Jones	(1) Prog., vit. E & thyroid	85%	3 or more
	(2) No therapy in indicated group	20%	3 or more
	(3) No therapy in group without indications	50%	3 or more
Javert	Psychotherapy, vitamins C, K, P	91%	3 or more

The salvage rates reportedly obtained in the management of habitual abortion are open to various interpretations because of the lack of uniform criteria in defining "habitual abortion" (Ferguson,[21] Grant[22]). Malpas[23] and Eastman[24] have afforded us some base line for estimating the salvage rate. So many different therapeutic regimens have improved the rate of salvage that the value of any one particular regimen over another may be questionable.

Recently, attention has been called to the possible psychologic value of the extra care and attention given to these patients as the remedial factor (Bevis[25]). Of 1820 pregnant women with a history of two or more previous abortions, observed and reported by nine different authors, King found that 61 per cent carried through to term regardless of the type of treatment or the lack of it.[26] King stated, "Treatment cannot be expected to change the outcome in more than a small percentage of cases. It should be directed toward correcting demonstrable abnormalities or deficiencies with due regard to psychologic factors including the patient's need for attention and support." A great variety of remedies have been used in the treatment of habitual abortion, with nothing in common except at high rate of success (TABLE 1). The British Medical Journal has offered the editorial suggestion that the good results may be due to the special attention bestowed upon these women. The importance of the psychologic factor in treatment should be determined by placebo studies.[27]

It is, therefore, with considerable misgiving that previously reported results are again presented.[3] It should be emphasized that, in this small series, only those habitual aborters have been included in whom a positive petechial test was obtained. The outcome in our first reported series was 84.6 per cent for those who had aborted twice, and 66.6 per cent (corrected value) in those who had aborted three to eight times. The series has been extended over the past two years, and it appears that the results remain at least as good. We are not unmindful that comparatively good results have been reported by others with or without special modes of therapy. We believe, however, that every habitual aborter should be studied prior to conception whenever possible, and that treat-

ment should be directed to whatever deficiencies are noted, capillary fragility among them. We also believe that hormonal therapy to support or enhance progesterone metabolism is worthy of trial. Whatever the curative factor may be, it is indeed rewarding to bring about a successful pregnancy in an habitual aborter. It must be acknowledged, however, that it would be imprudent to attach too great significance from the statistical point of view to the results obtained from the particular regimen of therapy recommended by us.

Conclusions

(1) If and when possible contributary causes are detected for habitual abortion during preconceptional studies, attempts should be made to correct them.

(2) If pregnancy is already established, a wide variety of remedies may or may not be employed. The physician might concentrate, with profit, chiefly on inspiring the patient with confidence in the successful outcome of the pregnancy.

(3) If capillary fragility is present, the use of bioflavonoids (hesperidin with vitamin C) may correct the capillary defect by modifying capillary permeability and vascular disturbance, whether in the skin, liver, decidua, or placenta and, consequently, influence the efficacy of established therapeutic procedures.

(4) If hormonal imbalance complicates the picture, it is within the realm of physiologic probability that efforts toward correction of the impaired progesterone production or utilization may elicit a favorable response. Progesterone, along with small doses of estrogen and/or chorionic gonadotropin, to enhance and maintain corpus luteum activity, may prove of value.

(5) The use of bioflavonoids and hormonal therapy, in the hands of this investigator, has considerably improved the salvage rate in his own cases of habitual abortion.

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Discussion of the Paper

Doctor Javert: I should like to make one point here that impressed me as I read the literature. Many writers describe a series of cases in which they have used hormones or stilbestrol or progesterone, and they evaluate their results in terms of these therapeutic agents. We find that, along with this therapy, they are using a lot of other things. I am thinking now of the prenatal vitamins and the calcium capsules that are used by almost every doctor in practice. The use of calcium began as a very humble substance by itself, usually as a pill, and I happily saw vitamin C added to it. In fact, the amount has now gone gradually from 10 mg. up to 50 and even 75 to 100 mg. and along with that we have other compounds, folic acid and vitamin B, as well as a few other substances that have been added to this calcium product. So I think, by and large today, it is a very rare pregnant woman that does not get some form of vitamin C along with her hormonal therapy.

Doctor M. H. Friedman (*Department of Physiology, Jefferson Medical College, Philadelphia, Pa.*): I should like to ask whether, in referring to hesperidin, purified hesperidin is meant or the hesperidin complex, or is it a general term referring to citrus flavine?

Doctor Robert B. Greenblatt: Doctor Martin would give us the best

answer, because he has been supplying us with the vitamin C for a long time. I am sure it is the citrus bioflavonoid we are talking about.

Doctor Martin: I think it extremely probable that the physiological activity of these molecules is shared by many such structures as flavones, flavanols. Doctor Friedman is referring specifically to this material. It is not a pure hesperidin. I do not believe, though, speaking from the standpoint of laboratory work, that there is any difference. By this I mean in enzymology. We cannot find any difference between the highly purified hesperidin and the crude form, but I do not think than any one means to imply that the physiological activity of the bioflavonoids is restricted to any single molecule. It is undoubtedly shared by many.

A RATIONALE FOR THE USE OF HESPERIDIN AND ASCORBIC ACID IN THE MANAGEMENT OF POLIOMYELITIS

By George J. Boines
Wilmington General Hospital, Wilmington, Del.

The purpose of this presentation is to attempt to establish a rationale for the use of hesperidin, a flavonone glycoside, combined with ascorbic acid as an adjunct in the over-all clinical management of the poliomyelitis patient.

We do not base the establishment of this rationale on conjecture, but rather on two concepts, namely the relationship of the capillary system to health and disease, and the established capacity of hesperidin combined with ascorbic acid to correct abnormal capillary fragility and permeability.

In developing our basis for the rationale of these combined substances in poliomyelitis, we must of necessity establish the importance of the capillary system, describe the histopathology found in poliomyelitis and, finally, show that we are justified in our speculation that hesperidin and ascorbic acid are necessary adjuncts in the treatment of poliomyelitis.

Let us consider the capillary system. When we view this system as filter beds where the essential function of the circulatory system is performed, we cannot help being impressed by its importance. Up to the stage of six weeks for the human embryo, these beds are the only absorbants, the primary absorbants, and they continue to take part in absorption throughout life.[1] It is true that subsequently other systems develop, such as the arachnoidal villi and the lymphatic vessels to assist in this function of absorption; still the capillaries continue to be important.

The smaller blood vessels play inportant and significant roles in the body, as evidenced by the following.[2] They function as: (1) part of the supporting structure of nervous tissue; (2) a mechanism for the maintenance of balance of blood plasma-cerebrospinal fluid relationship; (3) channels for supply of nutrition and oxygen; (4) a regulating mechanism (hypothalamic center) for control of visceral function; (5) a protective mechanism against disease; (6) conduits for the evacuation of decadent material from focal destructive lesions; and (7) sources of material for repair.

In the metabolism of nervous tissue, the small vessels play a vital part with respect to nutrition and tissue respiration. Too little is known of the mechanism of the effects of vitamin and mineral deprivation on the central nervous tissue to be specific as to their mode of action.

Normal function of the brain and spinal cord is dependent upon an intact vascular system. Any disturbances of the circulation affecting the nervous system result in symptoms of a magnitude commensurate with the number, the size, and the damaging effect of the resulting lesions.

Having a clearer understanding of the importance of the capillary system and its functions, we subscribe to the idea that "an intact capillary system means a solvent body." In other words, as long as the capillary system retains its integrity and has the capacity to carry out its normal physiologic function,

the body will remain in a state of solvency. On the other hand, if the integrity of this system is imperiled by infections, chemical or traumatic insults, the chances are that the body's solvency will diminish, and may lead to eventual bankruptcy; death.

The Histopathology in Poliomyelitis

Poliomyelitis is a systemic disease of virus etiology involving primarily the central nervous system and the voluntary muscular system.[3]

It has been suggested[4] that "many of the factors which enhance the severity of poliomyelitis or localize the paralysis, may operate through local vascular changes in the brain and spinal cord." On the basis of this concept, practical consideration should be given "to precautions necessary to reduce the chance of infection with poliomyelitis virus, and the physician should avoid inflicting on the patient any procedure which might seriously affect the blood supply of the nervous system and endanger the integrity of the blood-brain barrier."

A survey of the histopathological changes throughout the nervous system in 10 unselected cases by Baker *et al.*[5] revealed a most consistent and uniform involvement in all areas exclusive of the basal ganglia, suggesting some uniform method of spread of the infection, such as the vascular system. That the cerebellum is involved more frequently than not was also found,[6] again suggesting that "the widespread lesions within the cerebellum would point to a more diffuse dissemination of the disease process and would emphasize the part played by the vascular system in the spread of this illness."

The gross appearance of the central nervous system at autopsy reported[7] in each of four cases of fatal bulbar poliomyelitis in children in one family was essentially the same. There was severe congestion and swelling of the cerebral convolutions. Petechial hemorrhages were noted particularly in the left dentate nucleus, in the floor of the fourth ventricle, and in the pons, medulla, and the anterior horns of the spinal cord. Microscopic examination revealed much the same findings: very severe inflammatory reaction involving the spinal cord, medulla, pons, dentate nucleus of the cerebellum, and leptomeninges of the spinal cord and brain stem.

The importance of capillary damage in viral diseases was discussed by Lyon,[8] who concluded that "the capillary damages and their consequences in viral infections are important not only for the pathogenesis of these diseases, but also for their therapy."

The clinical picture of poliomyelitis may combine the features of an exceedingly complex and varied disease of nerve cells with those of a variable inflammatory process in the central nervous system.[9]

Reported observations[10] indicate that the permeability of membranes is altered during the course of acute infections. These observations are interpreted as indicating an alteration in the permeability of the capillary wall.

The presence of edema in the central nervous system affected by poliomyelitis has been adequately described.[2] That the edema of the central nervous system plays a dominant role in producing paralysis has been suggested.[11]

In the case of poliomyelitis, we agree that the magnitude of the capillary defect increases with the clinical severity of the disease. We believe that therapeutic profits in the clinical management of poliomyelitis can be realized more readily with the restoration of normal capillary integrity.

Vitamin C and Hesperidin—Review

According to Jungeblut,[12] "a study of the natural history of poliomyelitis suggests a vitamin C deficiency as one of the chief predisposing agencies." The susceptible individual appears to suffer from some deficiency in the operation of a biological mechanism concerned with the destruction in tissues of the virus, or with its extraneural fixation at the peripheral portal of entry.

It has been reported[13] that the vitamin C fraction of the adrenal gland was greatly reduced in monkeys killed or paralyzed by the virus of poliomyelitis. Identical findings were reported[14] in humans having died of various infectious agents.

Youmans[15] stated that "within a few hours after institution of adequate vitamin C therapy to correct an avitaminosis, histological evidence of bone improvement is obtained. Fibroblasts begin to form normal connective tissue and capillary buds are invading hemorrhagic areas."

Impaired nutrition and infection are factors which contribute to injury of capillary walls.[16] A deficiency of ascorbic acid is deleterious to intercellular cement substance binding the endothelial cells together. Infection causes an increased tendency to bleed, due to damage to the endothelial cells by soluble toxins of the infecting organism.

The accepted physiological action of vitamin C, according to Klenner[17] leads us to expect an antiedema effect in any given area.

"A striking phenomenon of vitamin C," reports Klenner,[18] "is the similarity of response, whether to correct pathologic processes due to a deficiency of this compound acting as a vitamin, or to destroy the ferments of microorganisms, acting as an antibiotic." This investigator observed increased capillary fragility to exist in all of his cases of poliomyelitis.

Vitamin C plays an essential role in the oxidation-reduction system of tissue respiration and contributes to the development of antibodies and the neutralization of toxins in the building of natural immunity to infectious diseases. McCormick[19] concludes: "There is an unusually broad spectrum of antibiotic action in this therapy (ascorbic acid) including all bacterial and viral infections."

The capacities ascribed to vitamin C are interesting, but, as a therapeutic agent, we agree with Bronte-Stewart[20] that "ascorbic acid by itself has no proved effect in combating any condition other than scurvy."

According to Scarborough,[21] a deficiency of vitamin C is not necessarily complicated by low capillary resistance. Rusznyak and Szent-Györgyi[22] reported that "in certain pathological conditions characterized by increased capillary permeability or fragility of the capillary wall, ascorbic acid is ineffective, while the condition can readily be cured by administration of extract of Hungarian red pepper or lemon juice."

A substance isolated from these concentrates was named "citrin"[23] and, on analysis, it was found to be a mixture of hesperidin and eriodictin.

Scarborough[24] studied the vitamin P activity of hesperidin and found this substance increased capillary resistance in man when given by mouth.

According to Szent-Györgyi,[25] "Vitamin P requires for its activity the presence of traces of ascorbic acid. In the entire absence of ascorbic acid, vitamin P is inactive."

After a review of the literature, Lindheimer *et al.*[26] stated that "a synergism of vitamin C and vitamin P is suggested by some studies, whereas others indicate that vitamin P acts by itself."

Rinehart and Johnson[27] concluded that vitamin P acts in conjunction with vitamin C in maintaining normal state of permeability of the capillary walls.

Warter and his associates[28] believe that hesperidin is essential for the absorption and retention of vitamin C, acting synergistically in maintaining normal capillary resistance.

That the utilization of vitamin C for the intercellular substance is dependent on the presence of hesperidin was suggested by Selsman and Horoschak,[29] who stated that "through some type of chemical reaction in the liver, hesperidin acting as a catalyst, vitamin C is combined with an intrinsic factor (protein), to form the intercellular substance."

Our experiences with hesperidin combined with vitamin C* extend over a period of four years and include approximately 400 patients. Abnormal capillary fragility was found in all of our severe acute polio cases. Six hundred milligrams of each agent were given daily in divided doses until the capillary integrity was improved. In 80 per cent of the cases, this improvement occurred in an average of five weeks. The patients experienced a sense of well-being and an increased appetite within one week.

It is our belief that the improvement in the integrity of the capillary system, following the administration of hesperidin and ascorbic acid, resulted in a definite enhancement in the patients' response to our over-all clinical management of poliomyelitis.

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* Hesperidin-C supplied through the courtesy of the Clinical Research Department, The National Drug Company, Philadelphia, Pa.

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Discussion of the Paper

Doctor C. W. Jungeblut (*College of Physicians and Surgeons, Columbia University, New York, N. Y.*): About 20 years ago, in our laboratory, the first attempts were made to examine the possible role of vitamin C and the function of vitamin C metabolism in experimental, but not in clinical, disease. That work began with the discovery that extremely small doses of ascorbic acid were capable of inactivating many times the fatal dose of the poliomyelitis virus. In fact, two or three milligrams were effective in inactivating 10 to 20 thousand fatal doses. It was the first demonstration that a physiological substance that is not an antibiotic and that is present in body fluids had the effect of producing an inactivation of the virus. After this discovery, it seemed a little irrational to assume that this inactivating effect could possibly be produced by injected vitamin C in infected animals, but we proceeded with this work and, in several studies, we obtained what seemed to be significant differences in the poliomyelitic attack rate in monkeys following cerebral injection, at that time, of the type 2 Akron strain, compared with those receiving vitamin C. This early work

was done with material which was furnished me by Merck and Company, of Rahway, N. J., following Doctor Szent-Györgyi's discovery. These were the first supplies of vitamin C made in this country from natural sources. I believe the product was mostly orange leaf, and it had a very yellow color. It was from this first product that we obtained the results mentioned. The synthesis of vitamin C was accomplished, and a white crystalline powder, crystalline ascorbic acid, came from Doctor Major's laboratory. We repeated our experiments with the pure chemical and the results were very much less significant and distinct than they had been with the impure product. I have been wondering, during these last 20 years, what that impurity was that gave us those interesting results.

It seems to me now, from the preceding papers, that there is at least a chance that we may have had flavonoid substances in combination with the ascorbic acid, and that, perhaps, it was these two substances that produced the results we saw at that time.

Since those early years, two other important facts have been added to our understanding of the pathogenesis of poliomyelitis that were not known at that time, and I think they may play a very important part in understanding what possible effects vitamin C might or might not have in producing beneficial effects in this disease.

First, it is now known that poliomyelitis virus is very constantly present in the blood stream before it reaches the central nervous system. In fact, many of us believe that the central nervous system phase is only a second phase following an early viremia. This was not known at that time, and the problem now is a very different one because, if one imagines virus circulating in serum and puts into that picture defective capillaries, it is not very far-fetched to imagine that capillary deficiency or immune deficiency might permit the virus to pass through leaky interspaces on its way to more susceptible nerve cells. That would make excellent sense if one tried to tighten the capillaries by giving a combination of substances that can do it.

Second, we know now that one of the outstanding bothersome pictures in poliomyelitis is the effect of pregnancy. It is now definitely known that, in age group 20 to 30, there is an attack rate of poliomyelitis approximately threefold that of the attack rate in nonpregnant women. This finding indicates very clearly that something happens in pregnancy that adds to the risk of paralysis. Again, Doctor Schwartzman has been able, experimentally, to simulate one of the excessive factors in pregnancy. By giving cortisone to animals injected with subinfective doses, he has been able to produce a tremendous effect. In fact, in animals infected with certain types of dosage, two or three milligrams of cortisone will convert the effect into a violent infection. From what we have heard now of the effect of ascorbic acid and hesperidin against DOCA, I think it is possible to imagine that, in combination, these two vitamins might have an effect against the excessive action of cortisone.

Finally, I should like to say that all clinical applications, of course, are difficult. I, myself, am more interested in laboratory data than in clinical data. Clinical data, as you know, are a written story that is often very difficult to

read, whereas laboratory protocols are fairly clear and simple. At least, you know what you are dealing with, but, on the clinical side, there are, in addition to the papers that Doctor Boines mentioned, at least two other good-sized series that have been published, both of them on vitamin C and ascorbic acid alone, and both series involve enormous dosages, going as high as one gram every four hours intravenously. Both of these series come from Basel, Switzerland. One was a series published by Bauer about 1951 or 1952, and the second, from the same institution, was published in 1955 by Doctor Zerl, a colleague of Bauer. Both of these men are good clinicians in the field of poliomyelitis. They came to exactly opposite conclusions. Bauer, basing his opinion on 25 to 30 cases, concluded he had unmistakably shown a clear-cut effect of his treatment. Zerl, in a somewhat larger series of 55 to 60 cases came to the conclusion that there was no difference in the extent of residual paralysis in treated or untreated cases. It seems to me that if one wishes to do something with vitamin C and poliomyelitis he should do it intelligently, in the way one would carry out chemotherapeutic treatment. One would not give terramycin to kill streptococcus without finding out whether there were blood levels and whether it is sensitive. It would be worth while for a group of clinicians to get together and treat cases intelligently, in order to determine whether there is a deficiency, utilizing urinary excretion, and to determine the dosage necessary to produce a spill-over effect of vitamin and, in that case, to build up a control series of treated and untreated cases.

Doctor Douglas Johnstone: That would be a work well worth following out. It seems that the practical point in handling poliomyelitis patients is to handle them early. If, as we know, the viremia comes early and the paralysis stage is only the tail end of the disease, which thus may be the crippling phase, then the use of the hesperidin answer, or the use of vitamin C, would unfortunately be applied too late. It is like treating children with gamma globulin to treat poliomyelitis. Theoretically, if one knew there was an epidemic, it might be very good, and of much prophylactic value to give some of these things to tighten up the venules, so that the virus would not get into the blood stream. Another point is that it is difficult to reconcile the relationship of the cortisone studies that have been mentioned with the fact that many malnourished people, at least people deficient in vitamin B, are said to be more resistant clinically to polio than well-nourished people.

Doctor Barnard: For some time, we have been obtaining a polymerized type of hesperidin. The polymerization is carried out by means of phosphoric acid. As a result we get macromolecules. These are anionic types of material that have come into considerable prominence because heparin is of very much the same type. We call them macroanions. We have studied this particular type of aggregated hesperidin and we find, as a matter of fact, that it is antiviral. It is a definite competitive inhibitor of the hemagglutinating viruses. While it is true that we have studied it in conjunction with the classically hemagglutinating viruses such as mumps, Newcastle's, we know that all viruses are potentially hemagglutinating viruses. Sometimes, however, it requires difficult techniques to bring out the hemagglutination because of the place of

attachment to the virus cells. Whether the virus is neurotropic or tissuetropic, it is still at the cholesterol lecithin site of the cell. The remarkable thing about macroanionic materials is that they are configured very much like hyaluronic acid or like the depolymerized types of hyaluronic acid that circulate as elevated transport mucoprotein in many of the conditions in which there is a dissolution of ground substance. It seems likely that phosphorylated hesperidin or any one of the aggregated hesperidins will prove to be like the antihistamines. They are competitive hyaluronidase inhibitors, but not histamine antagonists in the chemical sense. They will be antiphlogistic, and will prevent inflammation. They will become antiallergic, because every virus has very much the same configuration that a hemagglutinating antibody has. They will also turn out to be antithrombics. They will have heparinoid characteristics to a certain degree, and will become not only antihyaluronidase but antiribonucleases. In other words, there is some theoretical basis for believing that the competitive inhibition approach to the ribonuclease inhibition will be a much better approach to the problem of the proliferation of malignant neoplastic cells than the unfortunate fiasco of noncompetitive metabolites such as the antifols, the mercaptopurines, and the purine antagonists.

Doctor George J. Boines: We made no claims that the hesperidin-C treatment diminished paralysis, diminished the stiffness of the patients, or did anything else to the paralysis that we see in polio. We claim only that the petechial hemorrhages and the large number of petechiae which are found on the skin of patients with polio were reversed with hesperidin C. Now as to the controls, unfortunately, we do not, as clinicians, speak the same language as the laboratory people who work with rats and monkeys because we cannot segregate as many polio patients as they can without any treatment at all to see whether they get paralyzed and die while others are treated and get well. There can be no comparison on the use of controls. The over-all treatment that we use involves many other problems, but this is not the place to discuss them. But I wish to emphasize that hesperidin C is valuable to the patient, and that the earlier it is tried, the better the effect. Whether its use diminishes paralysis I cannot say, as that problem was not studied.

SUMMARY OF THE CLINICAL ASPECTS OF BIOFLAVONOIDS AND ASCORBIC ACID

By John B. Youmans
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While it is my purpose in this paper to summarize the clinical aspects of the bioflavonoids and ascorbic acid, it is not my intention to do so in the ordinary fashion, partly because of the very diffuse types of disease and disease states which have been the basis for these studies. Instead, I shall deal with some of the significant aspects of the studies which have been reported in this clinical field. I should like to emphasize that what I say applies to the clinical studies because these is a considerable difference, as it has been pointed out, between the studies in the test tube and the studies in the patient. The step is long and difficult to transfer one to the other, and I might give a wrong impression if some of my remarks were taken to apply to laboratory studies.

Other papers in this monograph have reported on clinical studies dealing with the effects of one or more substances in three rather distinct types of disease states. I use the term "disease states," because it is not clear except for two instances, polio and possibly rheumatic fever, that we are dealing with clear-cut, single disease entities. Rather we are dealing with symptoms which may be the result of several pathologic states and related causes. For this reason, we must seek some common denominator if we are to relate these clinical experiments to each other in the sense of establishing the mechanism of the action of the substances tested, namely, the bioflavonoids. It is worth while adding here that, in some of the experiments, the test substance has not been single or pure, so that there is introduced an additional one or more variables.

It is significant that the one common expression of disease or abnormality discussed in the papers included in this monograph is bleeding, in one form or another, whether into the skin, the mucous membranes, such tissues as the decidua, or such organs as the central nervous system. It is also significant that this bleeding is best, and perhaps only, explained on the basis of a loss of integrity of the capillary (venule) wall. The evidence of the action of the test substance, bioflavonoid, has been the prevention or cessation of bleeding.

Let us examine the possible basis or bases on which these effects might be expected. It can be assumed that vitamin C, at least, is necessary for the integrity of the capillary wall, since it is known that a deficient or ineffective supply is followed by clear evidence of capillary damage and bleeding from the capillaries. The same cannot be said for the related substances, the bioflavonoids, because, as far as I know, no satisfactory evidence of the effect of their lack has been demonstrated. However, some indirect evidence suggests that they may play such a role, possibly indirectly.

Vitamin C, then, and possibly the bioflavonoids, can maintain the capillary integrity in so far as their presence is required. The question is, can they maintain or restore it when it is threatened or affected by causes other than an

absence of themselves? It would seem so to me only if the mechanism by which other agents affected this integrity was related to the mechanism by which ascorbic acid and the bioflavonoids act. This may or may not be true.

This then leads to the conclusion that the bioflavonoids could act in one or more of the three following ways with respect to the effect of other disease:

(1) By maintaining a strong, normal capillary as far as it is concerned.

(2) By restoring the capillary to normal when its weakness is the result of a deficiency of these substances.

(3) By counteracting the effect of other factors which injure the tissues. This can be done by a direct or indirect mechanism not related to their primary function in relation to the capillary, or by resisting, with increased amounts, the action of the other agents against the flavonoids themselves.

All of the five clinical papers included here suggest one, or more than one, of these actions but, unfortunately, for one reason or another, they do not prove it, nor provide evidence of it. Perhaps this is not to be expected, from the nature of the studies. The degree of suggestibility varies with the various types of disease and nature of the clinical study. In all, a degree of pre-existing or acquired deficiency of ascorbic acid and perhaps other bioflavonoids can be expected. However, the amount and degree are difficult to estimate. Measures of ascorbic acid deficiency are not altogether reliable, especially at certain border-line levels. If some deficiency, however, is present, some improvement on such a basis is to be expected.

Evidence of another type of action is difficult to secure from clinical studies such as these. Levels of ascorbic acid excretion or concentration in the blood might well reflect the increased utilization of that substance to replace either destruction of it or its use in some other defensive action. Again, unfortunately, in those studies in which determination of levels were made (Javert-Greenblatt), there was not sufficiently close correlation of this with other findings to justify such a conclusion. Capillary fragility tests, in addition to other defects, have likewise failed to show correlation with findings indicative of deficiencies of ascorbic acid.

This forces us to depend primarily on the therapeutic result; *i.e.*, the effect of the administration of these substances on symptoms, mainly evidence of abnormal bleeding. This is, of couse, a difficult matter. In some cases, as in Greenblatt's study, the numbers are too small to be very reliable statistically. In others, the results are difficult to evaluate because of the necessity of employing a variety of agents in treating the illness, thus making it difficult to evaluate the effect of any one (Javert). In still others, the lack of consistency of disease in the test subjects has been a limiting factor. Those factors all interfere with the preciseness and clearness with which one can form conclusions. Now I should like to point out that it would be very desirable for us to devise other measures of the effectiveness or the action of these substances. We might agree that there is evidence, not conclusive but suggestive, and suggestive in various degrees, that these substances do have these beneficial results in the case of clinical medicine; but I think we need additional measures, additional indices involving some of the other chemical and biochemical actions

which have been described here to be applied in the case of clinical studies, so that we can come out with a more clear-cut understanding of the action and mechanism of these substances.

I should like to conclude with just a few remarks in general, the substance of which has already been stated, in some degree, by one or two other discussants.

In the field of medical research, so-called clinical research is often looked on with distrust and even disdain by research workers in the field of the medical sciences. It is curious and somewhat ironic that the reasons for this attitude are those features of research which make it difficult and should call for sympathy. These characteristics are adequate controls, adequate sampling, duration of observation, reproducibility, *etc.*, all features which are well known to all researchers.

It is true that these features are often lacking or inadequate in clinical studies, and that deductions and conclusions are sometimes made and drawn which pass beyond desirable limits. On the other hand, these characteristics of good experimental protocol are often, in fact, usually, difficult to secure when one is dealing with human subjects and human illness, and one is forced to dip more deeply into empiricism than one would like. It is for this reason that carefully designed and properly performed clinical experiments can provide some of the best examples of highly significant and important research in the field of medicine.

I should not want it to be inferred from what I have said that careless or inadequate research or incompetent research is to be excused or approved. I wish merely to point out that it is not often possible to be as precise in clinical research as in nonclinical experimentation.

PERSPECTIVES FOR THE BIOFLAVONOIDS

By Albert Szent-Györgyi
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In presenting the final paper in this monograph, I feel I ought to sum up what has been said and give account of where we are and whither we are going. Many interesting observations have been presented, and Doctor Martin has given us a most lucid review of the diverse actions of flavonoids. To complete this list, I should like to mention Doctor J. Kramar's paper, given last year at the International Physiological Congress at Montreal, Canada, which suggested that flavonoids have a part even in the hormonal balance influencing the relations of cortisone and the somatotropic pituitary hormone. Taking everything together, there can be little doubt that flavonoids are not only useful therapeutic agents in conditions of capillary fragility, but have many diverse actions in the animal body.

Such an activity poses an intriguing problem. My long-standing acquaintance with living matter has taught me to look upon it as a mechanism which, in its precision, greatly surpasses the finest Swiss watch. Capillary fragility means that this mechanism is out of order, and the idea of rectifying it with a decoction of orange peel seems to me like repairing a Swiss watch by driving a nail into it.

This absurdity can be eliminated by a philosophical outlook. In my eyes, there is but one living matter on this globe. However different their shapes, colors, and complexities, all living systems are but leaves of the same old tree of life and are based on the same common basic principles. There is no real difference between cabbages and kings. So, if the king's capillaries do not work well, possibly because one of their constituents is missing, and you can put these capillaries right again by an extract of the cabbage, then this means that you have set one precision mechanism right by taking out an identical screw from another similar precision mechanism, and the good fit only shows the close relation, the essential identity, of the two systems.

This picture resolves the contradiction, but involves an assumption for which there is no conclusive evidence; namely, that flavonoids are normal constituents of both animal and vegetable systems. Our whole outlook on the action of flavonoids depends on this question whether they are normal constituents of the animal cell. If they are, then we can suppose them to decrease capillary fragility because they replace a missing normal substance which the animal body itself was unable to produce. In this case, the flavonoids conform to the definition of a vitamin. However, if they are not normal constituents of the animal body, then we have to abandon the idea of a vitamin, and are faced simply with some accidental drug action which cannot claim a deeper biological interest.

Perhaps this division of substances into "vitamins" and "drugs" is too rigid and there is something between. There is no doubt that our body is dependent on an outside supply for the classical vitamins under any condition. But there also may be vital substances which our body can produce under normal condi-

tions but is unable to synthesize under exceptional circumstances, such as stress, infection, or the influence of some genetic shortcoming. In such a condition, our body would become dependent on an extrinsic supply. Such an assumption might explain why patients with nonthrombocytopenic hemorrhagic purpura are benefited by flavonoids. If these assumptions are correct, then purpura could be termed a "conditioned avitaminosis," and flavonoids termed "conditioned vitamins." Such a conditioned avitaminosis may be at the bottom also of other degenerative diseases. Schreiber and Elvehjem's[1] latest findings support such an assumption in relation to flavonoids. Similar ideas have been expressed by A. J. Lorenz, who used the word "synthetic" instead of "conditioned."[2]

So the cardinal question about flavonoid action is whether these dyes are normal cell constituents for the animal or not. Flavonoids, as suggested also by their name, are characterized by their very vivid yellow color, therefore it seems to be an easy matter to find them if they are there. As far as I am aware, efforts to demonstrate them in the higher animal conclusively have failed, suggesting a negative answer. However, caution is warranted. Carotenoids also are intensely yellow, yet they are used by the animal body as vitamin A, in a colorless form. In the case of flavonoids, a change in dissociation is sufficient to abolish color, and we might ask whether flavonoids are not present in the animal cell in the form of uncolored complexes.

Wishing to contribute more than words to this discussion, I have before me as I write a test tube which contains 1.5 g. of an intensely yellow substance which I extracted from animal tissues, from the thymus gland, in which it is present in an amazingly large quantity, of the order of 0.1 mg. per g. of fresh tissue, and in a colorless form, as part of a complex. It shows most reactions which characterize flavonoids. It is intensely yellow, as is also shown by its light absorption (FIGURE 1) in alkali at 400 mμ. On adding acid, the absorption shifts by almost 100 mμ to the shorter wavelength, below 400 μm, which means that the color disappears. Doctor T. A. Geissman, who was kind enough to take a look at this substance, called my attention to the fact that the acid absorption spectrum of quercetin is very closely similar to the spectrum of this substance under alkaline reaction. Most flavonoids give a dark color with Fe^{+++}, and so does this substance. Flavonoids give very insoluble compounds with polyvalent ions, such as Zn or Al, which are mostly more intensely colored than the free flavonoids. This complex formation, as shown by Clark and Geissman,[3] is due to a chelate formation, which is one of the most characteristic features of flavonoids. The Zn complex of this substance has all the earmarks of a chelate with a very high stability constant. Similar to other flavonoids, this substance is poorly soluble in water, its solubility being greatly increased by alkali. The situation, however, is not equivocal because the cyanidin reaction is negative, and the solubility of the substance is greatly enhanced by strong acid, which suggests either an exceedingly reactive oxygen, giving an oxonium, or, more probably, indicates the presence of nitrogen. Possibly, the animal body attaches an amino group to the flavonoid molecule

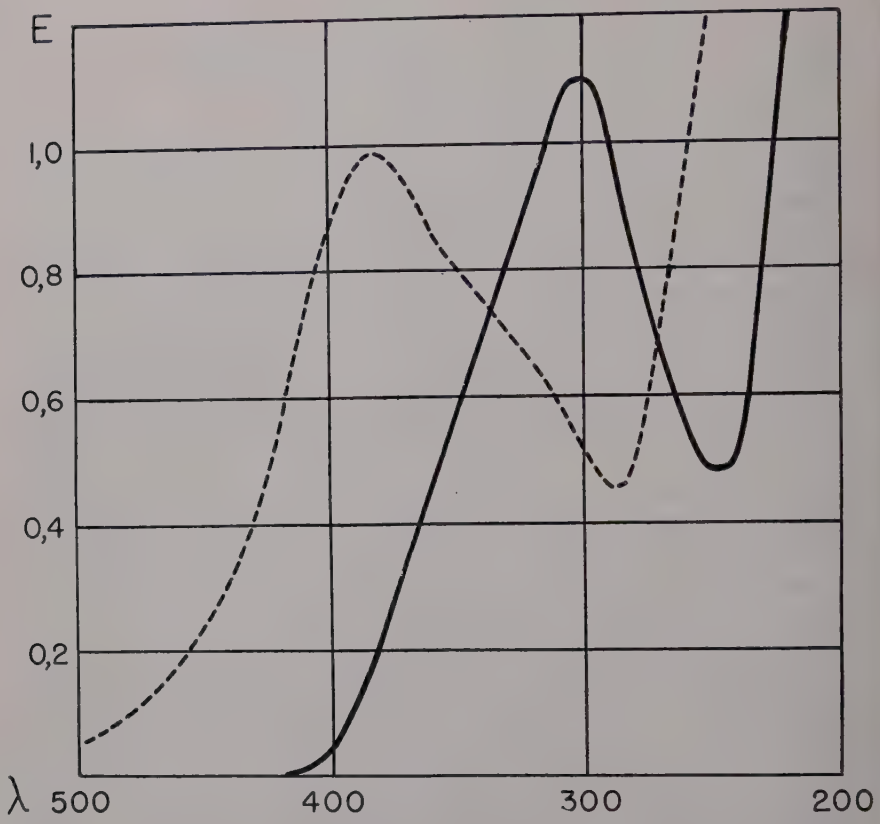

FIGURE 1. Extinction of the thymus substance at alkaline (broken line) and acid reaction (solid line)

to make it fit into its machinery. The answer can be expected only from the final structural analysis.

In any case, this substance again opens up the question whether flavonoids are normal cell constitutients of the animal body. My first experiments suggest that this substance is found in especially high concentration in the thymus gland, so it is not impossible that this dye will not only be found to be a flavonoid, but will also lead to a better understanding of the function of the thymus gland and the isolation of its hormone. My very preliminary experiments suggest that this flavonelike substance is an important ingredient of diverse, hitherto unknown biocatalysts.

The most interesting problem about any biologically active substance is that concerning the molecular mechanism of its action. In most cases, we cannot answer this question because we know too little about the other partner of the bargain, the living system on which the substance acts. So it is not surprising that this question has not yet been answered for flavonoids which, as judged by their quantity in the plant, must be one of the most cardinal biological agents. As a chemist, I am deeply impressed by the reaction of flavonoids with metals

while, as a biochemist, I am increasingly impressed by the central role metal atoms play in biological function. So it seems possible that the reactions of flavones with metals hold the key to the understanding of their biological function, while the flavonoid metal complexes may hold the key to a better understanding of the working of the machinery of life.

I regret that I must conclude with many questions asked and none answered, but I hope to leave the reader with the impression that flavonoids represent one of the most exciting, broad, and hopeful fields of biological inquiry, and I am glad to close on such an optimistic note.

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